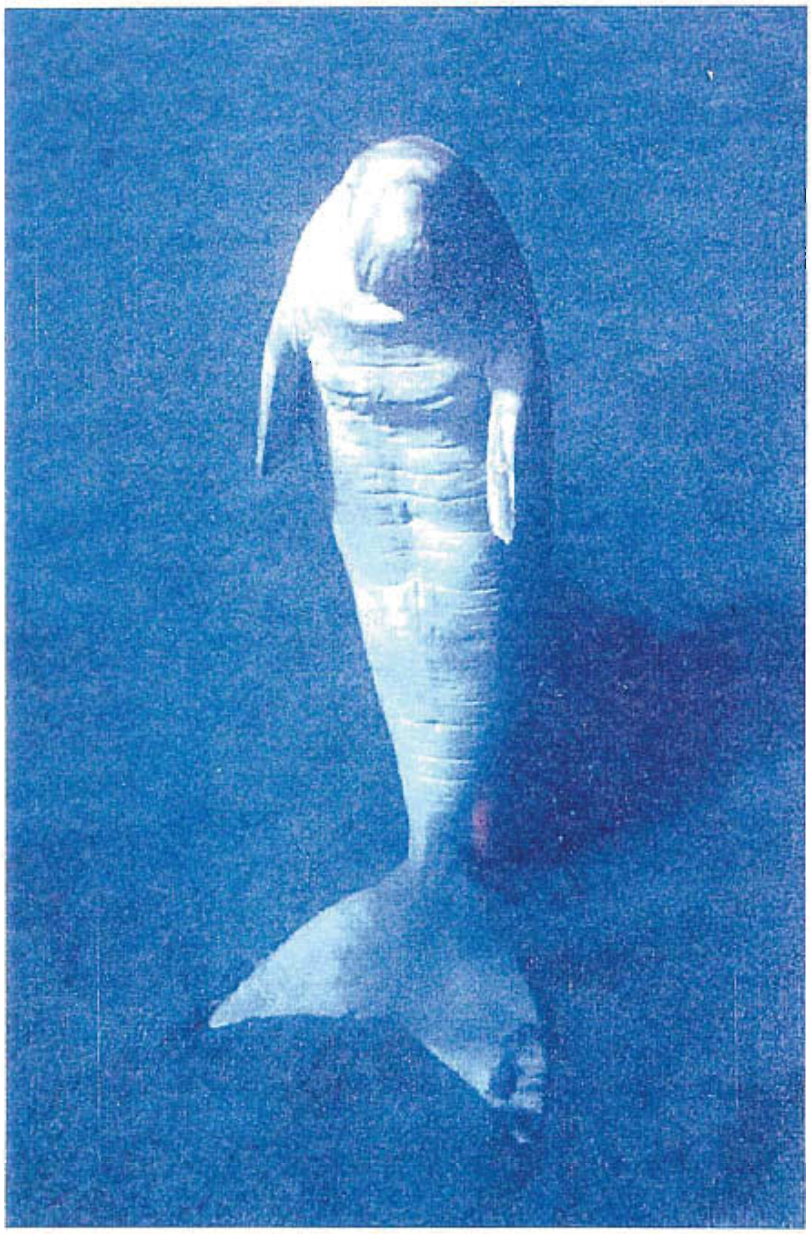
Using a Genetic Approach to Optimise Dugong (*Dugong dugon*) Conservation Management



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ABBREVIATIONS

A	adenine
Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degrees celcius (centigrade)
$CaCl_2$	calcium chloride
cm	centimetres
cpm	counts per minute
CSB	conserved block
DDT	dithiothreitol
D-loop	displacement loop
dmf	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
df.	degrees of freedom
E	east
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
eq(s).	equation(s)
et al.	and others
EtBr	ethidium bromide
EtOH	ethanol
g	gram
x *g*	multiple of the earth's gravitational field
G	guanine
H	heavy (strand of mitochondrial DNA)
HCl	hydrochloric acid
H_2O	water
HVR-1	hypervariable region 1
i.e.	that is
IPTG	isopropyl-D-thio-galactosidase
kb	kilo base
kcal/mole	kilocalories per mole
KCl	potassium chloride
km	kilometres (10^3 m)
L	light (strand of mitochondrial DNA)
LB	luria broth
lbs	pounds (one pound equals 0.4536 kilogram)
m	metres
M	molar (mol/l)
mA	milli ampere (10^{-3} A)
mg	milligram
Mg	magnesium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
mini prep	mini preparation

min	minute(s)
ml	millilitre (10^{-3} l)
mM	millimolar (10^{-3} mol)
mRNA	messenger RNA
mtDNA	mitochondrial DNA
Myr	million year
N	north
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram (10^{-9} g)
nm	nanometres (10^{-9} m)
OD	optical density
O/N	overnight
ORF	open reading frame
OTU	operational taxonomic unit
P^{32}	isotope phosphorus 32
PCR	polymerase chain reaction
PDA	piperazine di-acrylamide
PEG	polyethylene glycol
pH	negative logarithm to base 10 of the hydrogen-ion concentrations
pmol	picomole (10^{-9} mol)
Phe	phenylalanine
Pro	proline
PVP	polyvinylpyrrolidine
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	room temperature
S	south
scnDNA	single copy nuclear DNA
SDS	sodium dodecyl sulphate
s.e.	standard error
sec	second(s)
sq.in.	square inch
T	thymine
Taq	*Thermus aquaticus*
T4PNK	T4 polynucletotide kinase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	unit(s) (refers to enzyme activity)
U	uracil (pyrimidine base, constituent of RNA)
V	volts
VNTR	variable number tandem repeat
x	times
X-gal	5-bromo-4-chloro-3-indolyl-galactosidase
µCi	micro curie
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} l)
α	alpha
γ	gamma
ϕ	phi
3′	3 prime
5′	5 prime

GLOSSARY

allele one of a number of alternative forms of a gene that occupy the same specific position/locus on a chromosome.

allozyme one of a number of forms of the same enzyme which vary in electrophoretic mobility due to charge differences, specified by alleles.

bottleneck a sudden decrease in population density with a resulting decrease in genetic variability within a population.

cloverleaf secondary structure feature of DNA sequence composed of multiple hairpins.

conservative characters that change little during evolution.

DNA family set of genes with similarities in their nucleotide sequences, and which are thought to descend by duplication and subsequent variation from the same ancestral gene.

degenerative one amino acid can be specified by more than one codon.

Evolutionarily Significant Unit (ESU) where a population has adapted to local conditions and indicates a reservoir of unique genetic variability (Moritz, 1994a; 1994b; Moritz *et al.*, 1995).

Management Unit (MU) populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles (Moritz, 1994).

frameshift mutation which causes a change in reading frame as a result of the insertion or deletion of non-multiples of three consecutive nucleotides in a DNA sequence.

genetic drift random nucleotide changes in a gene not subject to natural selection.

genome library DNA library consisting of fragments of chromosomal DNA.

hairpin region of duplex structure DNA formed by base pairing between adjacent complementary sequences on the same strand, the unpaired bases between the sequences forming a single-stranded loop (hairpin loop) at the end of the hairpin.

haplotype specific DNA sequence.

haplotype cluster a distinct grouping of haplotypes on a gene tree.

haplotype diversity DNA sequence level analogue of heterozygosity (unlike nucleotide diversity, it does not require assessment of the magnitude of genetic divergence between haplotypes).

heteroplasmy more than one genetic type in an individual.

heterogeneity genotypic dissimilarity, descendent from different ancestral stock.

heterozygosity proportions of heterozygotes for a given locus in a population.

homoplasmy where DNA molecules are identical from one organ to another within an individual.

hot spot region of a gene where mutations preferentially occur.

introgression the gradual diffusion of genes from the gene pool of one species into another when there is some hybridisation between them as a result of incomplete genetic isolation.

junk DNA a vernacular term for the large amount of DNA in the genome that is composed of repetitive, non coding and intron DNA sequences, and appears to have no effect on phenotype (i.e., almost everything except for coding DNA).

L (light)-strand the inner circular strand of the (double-stranded) mitochondrial DNA genome, which is replicated second (to the heavy strand) and encodes fewer genes.

locus position of a gene on a chromosome.

nucleotide divergence nucleotide differences between populations.

nucleotide diversity heterozygosity at the nucleotide level (mean sequence divergence between haplotypes).

plesiomorphic ancestral (haplotype).

polymorphism stable long term existence of multiple alleles at a gene locus. By convention, a locus is polymorphic if the most common homozygote occurs at a frequency of less than 90% in the population.

polytomy where three or more branches (of a gene tree) meet at a node.

population is used to define the geographical location from which the dugong sample was collected.

promoter DNA region involved in and necessary for initiation for transcription.

redox mutual oxidation and reduction.

replacement amino acid change.

silent site neutral mutations, usually point mutations which change one codon into another specifying the same amino acid or a substitute amino acid which does not affect protein function, or mutations in a DNA region which has no genetic function.

site position of a nucleotide (base) in a DNA sequence.

substitution nucleotide change.

Tethyian seaway ancient sea between Laurasia and Gondwanaland.

transcriptional site point where DNA replication begins.

triplet three nucleotides equal one codon which translates to one amino acid.

Most of these definitions are from Lawrence (1989).

ABSTRACT

Distributed throughout the coastal tropical and sub tropical waters of the Indo-west Pacific, the dugong (*Dugong dugon*) is considered rare over much of its range and listed by the IUCN (1996) as 'vulnerable to extinction'. The largest numbers of dugongs are believed to occur in Australian waters. The main threats to dugong numbers are anthropogenic activities, such as accidental netting, habitat deterioration and Indigenous hunting. The primary objective of this study is to recognise intraspecific genetic subdivision for dugong management. Complementing ecological studies, these findings have immediate and practical relevance to the conservation management of the dugong.

Genetic material was sampled from various types of dugong tissue including skin, muscle, bone, and their faeces. Advances upon established sampling approaches for marine mammals include a biopsy system tailored to dugongs and the extraction, amplification and sequencing of dugong DNA from their faeces. Samples from approximately 250 dugongs were collected by carcass salvage, from dugongs hunted by Indigenous peoples, and by remote sampling of free ranging dugongs. Not all the samples collected were analysed, mainly because of low sample quality.

From dugong samples collected from Australia (n=92), West Indian Ocean (n=4) and Asia (n=7), three genetic markers were investigated: the cytochrome *b* gene and control region of mitochondrial DNA (mtDNA), and microsatellites. Cytochrome *b* proved to contain insufficient variation for an interpopulation comparison, whereas a block of DNA sequence (194 bases), positioned 5′ in the control region of mtDNA, was identified as suitable for interpopulation comparison because of its high variation. A substantial foundation for the development of microsatellite markers for future research was established by this study. In addition to recognising a six base repeat located 3′ in the control region of mtDNA, five GT-AC imperfect microsatellites were sequenced from a dugong genome library. The application of microsatellites was not developed further than their location and characterisation.

The hypervariable region 1 of mtDNA was sequenced for a total of 103 dugongs, as well as in an outgroup, the Florida manatee (*Trichechus*

manatus latirostris). Among the dugong sequences, 39 variable sites and 37 haplotypes (specific DNA sequences) were found. Phylogenetic trees constructed from the mtDNA haplotypes showed three clusters: West Australian, East Australian and Asian. These haplotype clusters suggest that Asian and East Australian dugongs are more closely related to each other than either group is to the West Australian dugongs. Surprisingly, haplotypes of West Indian Ocean dugongs are extremely similar to the haplotypes of dugongs from East Australia despite their large geographical separation. This suggests that the West Indian Ocean and East Australian dugongs shared a more recent ancestor compared to the Asian or West Australian dugongs. The geographical range of the Asian mtDNA haplotypes does not overlap with the Australian haplotypes. The two Australian mtDNA haplotype clusters overlap geographically in the Great Barrier Reef region.

The intraspecific genetic partitioning of dugong populations from Australia, Asia, and the West Indian Ocean, can be partly explained in terms of historical geography. The distinction of the two Australian mtDNA haplotype clusters may be attributed to the Torres Strait (land bridge) acting as a periodic barrier to dugong movements during the Pleistocene low sea level phases. It is of particular interest to management that West Australian haplotypes have such a limited spread to the south and east Australian dugong range. Similarly, East Australian haplotypes do not extend north and west along the Australian coast beyond Torres Strait. Considering the dugong's potential for dispersal, the spread of the two Australian haplotype clusters is remarkably limited.

Dugongs have a high intraspecific genetic diversity (average nucleotide diversity = 3.425, and haplotypic diversity = 0.766), and a rate of evolution (suggested to be 2% per Myr per lineage for the HVR-1) that is comparable to most land mammals. The suggested low level of female mediated gene flow between neighbouring populations indicates that successful recolonisation of a depleted area may be extremely slow. For conservation management of the Australian dugong, populations should be treated as distinct units with some degree of overlap. To maintain continuity among these populations, and to preserve the existing high intraspecific genetic diversity, management initiatives should be directed at limiting further fragmentation of the dugongs' range.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 DUGONG - CONSERVATION REQUIREMENT

The dugong (*Dugong dugon*) is a large marine mammal with an extensive coastal range, a long generation time (see Marsh, 1980), low fecundity (Marsh, 1995), and a specialised diet (Johnstone & Hudson, 1981; Marsh *et al.*, 1982; Lanyon *et al.*, 1989). These biological and environmental factors suggest that the dugong is a species with a high risk of extirpation (Millsap *et al.*, 1990).

Dugongs are found in the sub-tropical and tropical, coastal and island waters of over 40 countries between east Africa and the Solomon Islands/Vanuatu. The dugong's distribution overlaps that of the seagrass on which they feed (Poiner & Peterken, 1995). As a consequence of their coastal distribution, dugongs are subjected to pressures from anthropogenic activities. As a seagrass specialist, the dugong's existence is further compromised by its limited capacity to adapt to these pressures. In Australia, dugong have been subjected to economic exploitation in the past (e.g., Peterken, 1994) and continue to be hunted by Indigenous peoples (Johannes, 1981; Johannes & MacFarlane, 1991).

On the basis of the Red List Categories of Threat (IUCN, 1996), the dugong is listed as 'vulnerable' to extinction under criteria **A1cd**, which are applicable at the global scale as follows:

Criterion **A1:** A population reduction of at least 20% in 10 years or three generations, whichever is the longer. Generation time is defined by the average age of mature adults in a population, which is estimated to be 30 years for the dugong (see Marsh, 1980 for age distribution). Therefore, the time frame for which reduction in dugong numbers should be considered, is 90 years.

c. A population decline projected or suspected in the future based on: a decline in area of occupancy, extent of occurrence and/or quality of habitat.

d. The actual or potential levels of exploitation.

The legitimacy of applying the IUCN criteria at a regional scale is controversial (Gärdenfors, 1996). This is because of the unknown likelihood of recolonisation from one region to another, and the absence of knowledge on the intraspecific structure of the dugong. As a result, authorities are uncertain of the significance of local depletions, such as has been observed in the southern Great Barrier Reef, and in Hervey Bay (Queensland, Australia). Acknowledging the existence of any intraspecific genetic structure will benefit the development of a conservation plan for the dugong by enabling predictions of the likelihood of recolonisation and by recognising genetically unique regions. Genetic markers are an effective approach to recognise intraspecific units. Once intraspecific genetic units are recognised, it becomes possible to identify factors which may affect the genetic structure of the dugong. Intraspecific units can be compared to obtain measures of within-unit genetic diversity and the degree and rate of any interaction among units. Approximate average population size of reproductive adults over recent evolutionary time, can also be suggested from the level of genetic variability.

Mitochondrial DNA (mtDNA) evolves rapidly, making it suitable as the genetic marker for population level studies such as this. In addition to the information provided by mtDNA, spatial information can be used to recognise intraspecific structure for the dugong. A representative subset of haplotypes (unique DNA sequences) can be used as the basis for determining the level of intraspecific genetic structure.

Dugongs have considerable potential for dispersal, as individuals can move hundreds of kilometres in a few days (Marsh & Rathbun, 1990). However, large scale mass migrations (e.g., like those of the humpback whale, Chittleborough, 1965) have never been reported. Considering the extensive range of the dugong relative to known individual movements, and the lack of evidence for mass migration, reproductive isolation of populations is likely, and as a result, some genetic subdivision might be expected. Furthermore, anecdotal reports and morphological differences (Spain & Marsh, 1981; Preen, 1989b) suggest within-species genetic subdivision of the dugong is likely to exist.

This study aimed to:

- Establish effective molecular markers for a population level study of the dugong.

- Use these genetic markers to recognise intraspecific structure for the dugong.
- Assess within-unit genetic diversity, and between-unit genetic divergence.
- Present a phylogeographic model, and relate it to dugong distribution and dispersal.
- Make management recommendations based on the genetic findings.

The information on intraspecific genetic structure produced by this project is central to the design of an effective management regime for dugongs. In addition, the project has potential to act as a model for the use of molecular techniques in addressing ecological questions for other coastal species.

1.2 FORMAT OF THE THESIS

This thesis consists of 12 chapters. Chapters 1 and 2 review current understanding of dugong biology and behaviour, essential for the interpretation of genetic data. These chapters also highlight the requirement for dugong conservation and an effective management regime. The evolution and historical distribution of the dugong are detailed in Chapter 2, to assist in the interpretation of the genetic patterns. Various types of molecular markers were reviewed to justify my choice of markers to investigate intraspecific structure for the dugong.

Chapters 3 and 4 describe the approach I used to develop a tissue bank, and genetic methods, respectively. A wide range of DNA sources was explored, from faeces to the biopsy sampling of free ranging dugongs (Chapter 3). The methods for DNA extraction, analysis and sequencing are detailed in Chapter 4.

Results of sequencing the dugong mtDNA are presented in Chapter 5 (cytochrome *b* gene), Chapter 6 (control region), and Chapter 7 (hypervariable region 1). Intraspecific genetic structure for the dugong was identified using data from the hypervariable region 1 of mitochondrial DNA in Chapter 7. The application of statistical methodology to estimate substitution rates and predict divergence times between nominated

geographic and genetic units is described in Chapter 8, which also includes estimates of effective female population size and female 'migration' rates.

Chapters 9 and 10 outline research that utilised the material available in this study, but did not directly contribute to recognising genetic substructure for the dugong. Chapter 9 describes the results of a pilot study to develop microsatellite markers. Chapter 10 details the application of information from this study to wildlife forensic science, as dugongs are a protected species within Australia.

In Chapter 11, I develop a phylogeographic model based on genetic structure (Chapter 7) and substitution rates (Chapter 8). In the General Discussion (Chapter 12), the general implications of this study for dugong management are developed, in addition to suggestions for future research.

1.3 PRINCIPAL FINDINGS

- The hypervariable region 1 of mtDNA sequence from 103 dugongs from Australia, West Indian Ocean and Asia indicated three haplotype clusters corresponding to geographic regions:
  1) Asian
  2) West Australian
  3) East Australian.
- The geographical range of Asian mtDNA haplotypes does not overlap with Australian haplotypes.
- The geographic ranges of the Australian mtDNA haplotype clusters overlap in the region of the Great Barrier Reef. The distinction of the two Australian mtDNA haplotype clusters may be attributed to a Torres Strait (seaway between Papua New Guinea and Australia) acting as a periodic land bridge barrier to dugong movements during the Pleistocene low sea level phases.
- West Indian Ocean mtDNA haplotypes are very similar to the haplotype cluster of East Australia, suggesting a recent common ancestor.
- Subdivision among sample localities is suggested within each haplotype cluster for the Great Barrier Reef region.
- Suggested female mediated gene flow (Nm) between neighbouring populations in Australian waters, ranges from 0.47 to 6.17 migrants

per generation (30 years). This suggests that successful recolonisation of an area by dugongs following local extinction would be extremely slow (i.e., take several hundreds of years).

- Dugongs generally have a high within-unit and intraspecific genetic diversity, and rate of evolution, compared to other marine mammals such as cetaceans and pinnipeds. To maintain this genetic diversity, it will be important to avoid fragmentation of the dugong's Australian range.

CHAPTER TWO

USING A GENETIC APPROACH TO OPTIMISE DUGONG MANAGEMENT

The development of a genetic basis for the management of the dugong (*Dugong dugon*) is the primary objective of this study. This chapter reviews the terminology used to define intraspecific units as a basis for management. Non genetic and genetic markers used in this study are discussed to justify why the particular genetic approach was chosen. Also reviewed is the current understanding of dugong biology and behaviour, essential for the interpretation of genetic data. Taxonomy, evolution and historical distribution of the dugong are detailed to assist in the interpretation of the pattern of genetic variation, and the estimation of the genetic mutation rate. The biological and environmental factors that suggest that the dugong is a species at high risk of extirpation are considered. This chapter concludes with predictions about the genetic population structure of the dugong.

2.1 INTRASPECIFIC UNITS FOR MANAGEMENT

According to Baverstock *et al.* (1993) the primary role of conservation biology is to retain evolutionary diversity, flexibility, and as a result, long term viability of the species of interest; that is, to maintain the full array of differently adapted geographic variants within a species (Moritz, 1994b). In this context, an important aspect of conservation management is to recognise any subdivision (i.e., existence of distinct units) within the species, and the geographic scale of these units such that they can be monitored and managed (Baverstock *et al.*, 1993). Many authors employ an eclectic approach to the definition and terminology of intraspecific units for economic and/or conservation management. Intraspecific units may be defined by life history, morphology, behaviour, genetics, geography, or a combination of these (Barlow *et al.*, 1995; Moritz *et al.*, 1995). The ways in which each term has been used in the literature can be quite variable. The mode in which I have used terms for intraspecific units in this study are summarised in figure 2.1.

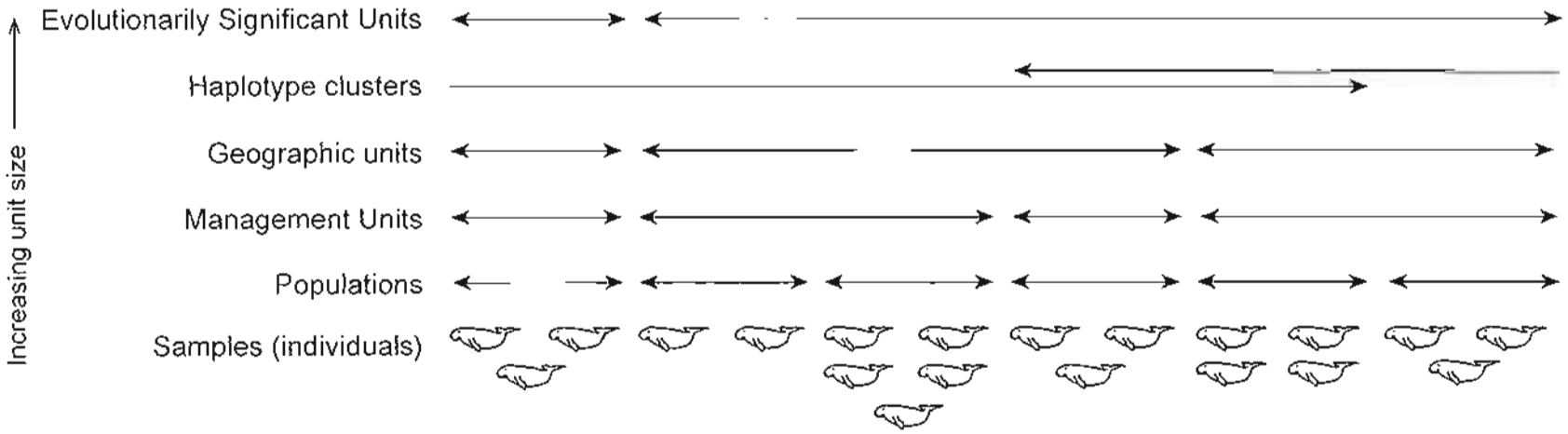


Figure 2.1: Subspecific units: terminology and definition. In this manuscript, a **sample** is an individual dugong from which material was collected. A dugong population refers to a number of sampled individuals from the same locality, and are defined by geography only. **Management Units (MUs)** are biologically contiguous units, and should be treated independently for conservation management. An MU differs genetically from a geographic unit in that an MU is defined by the relative difference in the frequency of haplotypes, whereas a geographic unit can be defined by the distinctiveness of haplotypes (as well as by their relative frequencies). **Geographic units** refer to units defined by haplotype divergence and frequency, and geography. There is no significant genetic difference among populations within a geographic unit. A **haplotype cluster** is grouping of haplotypes on a gene tree, determined by haplotype similarity at the nucleotide level. **Evolutionarily Significant Units (ESUs)** are indicated by clusters on a haplotype phylogeny, and do not overlap in a geographic area, whereas haplotype clusters can overlap. The distinctiveness of an ESU is dependent on the length of time in which its members were isolated from other populations and diverged under the influence of evolutionary processes.

2.1.1 Population

Various authors have used the term 'population' in the following ways to include: 1) all members of a species, 2) all members of a species in a defined area, or 3) a group of animals being studied (i.e., 'my study population') (Baverstock *et al.*, 1993). In this study, when the term 'population' is used in reference to dugongs, it indicates sample locality.

2.1.2 Deme, Subpopulation, Race

I have not used these terms because I considered their use to be inappropriate in the context of this study. A 'deme' is defined by Baverstock *et al.* (1993) as a group of individuals that exchange genes at random, that is, a genetically contiguous unit. 'Deme' approximates the definition of a genetic 'stock' (following section). A population can refer to individuals within an area of any size. Therefore the term 'subpopulation' is confusing. 'Race' suggests permanent morphological traits of a group of individuals, and is not a popular term in conservation management.

2.1.3 Stock

The usage of the term 'stock' is typically loose, subjective, and inconsistent. In addition, there is no universal consensus about how unique and isolated a population should be before it is called a stock. In fisheries management, the aim of recognising fish stocks is to ensure that they are harvested in such a manner that genetic diversity is maintained (Larkin, 1981). A fish stock is a genetically (or otherwise) distinct population with a common breeding ground (Larkin, 1981). Under this definition, fish from a single location (i.e., population) may be composed of several stocks (Larkin, 1981). The same is true of green turtles (*Chelonia mydas*), from a single feeding area (Karl *et al.*, 1992; Encalada *et al.*, 1996).

As some marine mammals are harvested as a resource (not unlike fish), the term 'stock' is used to describe units within-species as a basis for conservation management. A stock should be maintained as a functioning element of the ecosystem. According to the *US Marine Mammal Protection Act 1972* (MMPA), a stock is the fundamental unit of legally mandated conservation efforts, and refers to sympatric animals that interbreed. Barlow *et al.* (1995) states that stocks should be based on the smallest groupings which are: 1) biologically reasonable (supported by genetic and/or other

biological evidence), and 2) are practical from a management perspective. In the absence of sufficient information, a stock should be defined to include only the area from which marine mammals are taken (i.e., the area in which a specific fishery is operating) (Barlow *et al.*, 1995). Because of the inferences associated with the term stock, it will not be used in this study to describe a within-species unit. However for the sake of comparison, in the General Discussion I relate the intraspecific units defined in this study to a ranking system presented by Dizon *et al.* (1992), which clarifies the definition of a stock:

- *Category I* stocks exhibit a high level of geographic and genetic discontinuity with other stocks.
- *Category II* stocks are similarly characterised by genetic discontinuity, but have weak geographic partitioning.
- *Category III* stocks are the converse of *II*, with little genetic differentiation between clearly separate geographic units that are likely to be reproductively isolated.
- *Category IV* stocks are characterised by extensive gene flow and no subdivision by extrinsic barriers.

2.1.4 Distinct Population Segment (DPS)

Non-evolutionary reasons to ascribe conservation value to a particular population include its demographic, ecological, behavioural, economic, and aesthetic characteristics (Dizon *et al.*, 1992; Moritz, 1994b; Pennock & Dimmick, 1997). Traditionally, any of these reasons could be used to define 'distinct population segments' (DPSs) with the intention of conserving biodiversity (Pennock & Dimmick, 1997). Recently, the *United States Fish and Wildlife Service* and *National Marine Fisheries Service* proposed a policy that refined the DPS as a unique evolutionary entity, compromising management efforts because the role of demographic and behavioural data would be reduced (Pennock & Dimmick, 1997). Because of the political controversy as to what accurately defines a DPS, this study avoids making recommendations for DPSs, or using the term.

2.1.5 Subspecies

"A subspecies is an aggregate of local populations of a species, inhabiting a geographic subdivision of the range of the species, and differing taxonomically from other populations of the species" (Mayr, 1966). Subspecies may differ "taxonomically" on the basis of morphological,

genetic and biochemical characteristics, habitat (or host) range. Members of different subspecies within a species, can still interbreed, but are completely separated, geographically or otherwise. This definition resembles that of a stock. I will not use subspecies as a term to describe within-species units in this study, although previously proposed subspecific units for the manatee and dugong will be reviewed in section 2.5.

2.1.6 Evolutionarily Significant Unit (ESU)

Evolutionarily Significant Units (ESUs) can be recognised as monophyletic clusters on mitochondrial DNA (mtDNA) phylograms, and show significant divergence of allele frequencies at nuclear loci (Moritz, 1994a; 1994b; Moritz *et al.*, 1995). ESUs therefore, emphasise historical population structure rather than current adaptation and short-term conservation requirements. (Moritz, 1994b). Defining ESUs ensures that evolutionary heritage is recognised and protected, and that the evolutionary potential inherent across the set of ESUs is maintained (Moritz, 1994a; 1994b; Moritz *et al.*, 1995).

2.1.7 Management unit (MU)

Management units (MUs) are recognised as populations with significant divergence of haplotype (allele) frequencies at nuclear or mitochondrial loci, irrespective of the phylogenetic distinctiveness of the haplotypes (Moritz, 1994b; Moritz *et al.*, 1995). That is, populations that do not show reciprocal monophyly for mtDNA alleles, yet have significantly diverged in allele frequency, are significant for conservation in that they represent populations connected by such low levels of gene flow that they are functionally independent (Moritz, 1994b; Moritz *et al.*, 1995). MUs are identified to aid short-term management of ESUs (Moritz *et al.*, 1995). Moritz (1994b) suggests that the term 'stock' be restricted to short-term management issues (e.g., harvesting) and in relation to genetics, be treated synonymously with MUs. In this study, an MU is considered to be the smallest unit for management and may consider other factors in addition to genetics.

2.1.8 Haplotype cluster

A haplotype is a specific DNA sequence which can effectively be considered as an allele. A haplotype cluster (or genetic unit) indicates a genetically

similar grouping in a phylogeny. If a haplotype cluster were geographically monophyletic, it would equate to an ESU.

2.1.9 Phylogeographic and geographic units

In this study, a geographic unit is defined by a genetic (haplotype) grouping correlated with a geographic region. These units may have overlapping haplotypes. A phylogeographic unit is a geographic unit that does not have haplotypes that overlap with any other unit.

In this study, the main difference between geographic and management units, is that a geographic unit requires a genetic distinction, whereas a management unit would not.

2.1.10 Terms and their usage in this study

In summary, terms used in this study for intraspecific units are defined as follows:

- **Evolutionary Significant Units** (ESUs) are monophyletic clusters on mitochondrial DNA (mtDNA) phylograms.
- **Management Units** (MUs) address the current population structure, haplotype frequencies and short-term management issues (Moritz, 1994b).
- A **geographic unit** is considered as a larger unit than a MU (figure 2.1) and is based on haplotype divergence as well as differences in haplotype frequency. The term geographic unit is used in this thesis to indicate a unit which is defined genetically and geographically. Under this definition, a geographic unit may include more than one population.
- A **haplotype cluster** refers to a grouping of genetically similar DNA sequences in a mtDNA phylogeny.
- A dugong **population** is defined as the site of sample collection. It refers to a group of dugongs in a geographic area (only) (figure 2.1). For example, all dugongs sampled from Hervey Bay, Australia are defined as representing a single dugong population. When the term population is used and does not specifically refer to dugong sample locality, it should be considered as 'individuals of a species within a geographic area'. A population is viewed as smaller than a geographic unit. That is, there can not be more than one geographic unit within a population of dugongs.

2.2 STUDYING MARINE MAMMALS: NON GENETIC and GENETIC APPROACHES

2.2.1 Non genetic methods to identify units within-species

The choice of method employed to identify subdivision within a marine mammal species should be based on considerations of reliability, cost and time needed to gain the required discrimination capacity (i.e., ability to distinguish between individuals, families or populations) (Amos & Hoelzel, 1990).

Tracking (section 2.4.2) and tagging dugongs enable the researcher to obtain information on the fine scale habitat use of individual animals. If units for management were defined on the basis of the range of individual dugong movements, it is possible the species may be mismanaged as the question of the level of interaction between populations (i.e., are they interbreeding?) is not addressed. Units for management may exhibit distinct regional morphological characteristics (section 2.5), which are more indicative of interbreeding populations than tracking individuals. It is possible that morphological characteristics are environmental rather than genetic although, if the morphometric differences are consistent, this would suggest restriction of gene flow among populations in a species with a life cycle like the dugong (although not for species with planktonic larvae).

Information regarding individual dugong movements (section 2.4.2) has been obtained by observation from either a vessel or the coast, or during aerial surveys (table 2.1). These methods have also been used with varying success to study dugong behaviour and social structure (Anderson & Birtles, 1978; Heinsohn, 1981; Hudson, 1981b; Marsh & Lefebvre, 1994; Anderson, 1995b) and to obtain a local census (table 2.1). Observation is less reliable when water is murky or deep, and confusing when dugongs are at high density.

Photographing (or recording) freeze brands, scars and colour variation to identify individuals and map their movements and behaviour, and maintenance of these records, is a method that operates well if a marine mammal population is small and there is support from a network of volunteers and workers. A photo-identification system for Florida manatees run by the *Sirenia Project* in Gainesville, Florida, is such a

Table 2.1: Summary of non molecular markers. Cost and benefits of the various marking systems used on the Florida manatee and dugong.

Method and Application		*Florida manatee*	*Dugong*
Aerial survey. Used to obtain a census. Also informative with respect to distribution & behavioural observations.		The most accurate and feasible way of obtaining manatee population sizes (Ackerman, 1995).	The most accurate and feasible way of obtaining dugong population sizes (section 2.4.4; table 2.3).
Radio & satellite telemetry. Effective for tracking individual movements. Can also give some indication of population structure.		An informative and effective means of obtaining individual manatee movements (Reid *et al.*, 1995)	An informative and effective means of obtaining individual dugong movements (section 2.4.2; figure 2.2)
Photo-identification. Individual identification and behaviour.		A comprehensive identification system used with great success by the Sirenia Project, Gainesville, Florida. The photoidentification catalog has been useful for estimating: survivorship, movements, and reproduction traits (Beck & Reid, 1995).	As dugongs are more mobile, evasive and inhabit areas which are difficult to access, this approach is difficult, particularly when volunteer support is low, and dugong numbers are high. Not an effective means of obtaining a census. Some behavioural information has been gained from a study in Shark Bay, Western Australia (Anderson, 1995a).
Visual Tagging. In conjunction with mark & recapture, gives some indication of individual movements and enables individual identification during behavioural observations.	*tailstock belts*	No record.	Apparently 'trialed successfully' on dugongs of Papua New Guinea (Hudson, 1981b). Hudson (1981b) also suggested a highly visible buoy (for observation) attached at the end of 60 m rope, a suggestion which is discouraged as the rope could easily entangle posing a hazard to the dugong.
	spaghetti tags	No record.	Apparently 'trialed successfully' on dugongs of Papua New Guinea (Hudson, 1981b). Presently used on dugongs in south east Queensland by C.J. Limpus (pers. comm.). No data have been reported using this tagging method.
	freeze branding	Commonly used to individually mark captive Florida manatees (e.g., Homosassa Springs, Florida (pers. observ.)). Also see Beck & Reid (1995).	Proposed but not practised on dugongs in Papua New Guinea (Hudson, 1981b).
	paint stick	No record.	A solitary female was marked with a livestock-marking crayon, followed and her behaviour recorded (Anderson, 1982).
Observation. From the coast, marine vessel, or aeroplane. Enables study of behaviour. Used in conjunction with photo-identification and/or tagging.		For example see Beck & Reid (1995).	Anecdotal data provides much of the data base for dugong numbers (particularly outside Australia & Torres Strait). Also a number of behavioural observations on feeding, surfacing and diving.

program. Recognition of individual manatees is facilitated by virtually every manatee being scarred from boat propellers. Hudson (1981b) expressed interest in freeze branding dugongs as a visual tag, but the method has apparently not yet been employed. Other tagging methods have been tried on dugongs including tailstock belts and spaghetti tags (Hudson, 1981b; Limpus, personal communication). However, these methods have not provided any published information to date (table 2.1). Visual tagging programs are effective only if a high proportion of the animals in a population can be recognised. The limitations of visual tagging increase with population size. Most dugong populations are too large for visual tagging to be effective. The application of photo-identification to map individual dugong movements is not informative on a scale greater than 'extremely local'. Regular sightings of individual dugongs in Shark Bay (25°25'S, 113°35'E) were possible in embayments where dugongs were relatively sedentary. However these individuals were never resighted in open water (Anderson, 1995b).

Physical tagging methods require the marine mammal of interest to be captured and handled with relative ease. Capturing the animal is unavoidably intrusive, and may alter subsequent behaviour affecting the likelihood of recapture (Amos & Hoelzel, 1990). Capture can result in dugong mortality (Heinsohn *et al.*, 1976; Marsh & Anderson, 1983; Marsh, 1989). Other difficulties regarding the attachment of artificial tags on marine mammals (and their apparent loss at an unknown rate) are discussed by Amos & Hoelzel (1990).

Non genetic methods, in particular telemetry (section 2.4.2), have revealed information about dugong behaviour, social structure and movements. Morphological characteristics (section 2.5) also suggest the existence of intraspecific subdivision of the dugong. However, none of these methods provides the quantity and quality of information required to recognise the intraspecific structure required for effective dugong management.

2.2.2 Genetic markers to identify intraspecific units

Genetic markers are unsurpassed in their ability to reveal intraspecific units. For example, they would be able to verify if those dugongs that travel great distances (section 2.4.2; figure 2.2) interbreed with the neighbouring populations they visit. Genetic markers also provide information at other

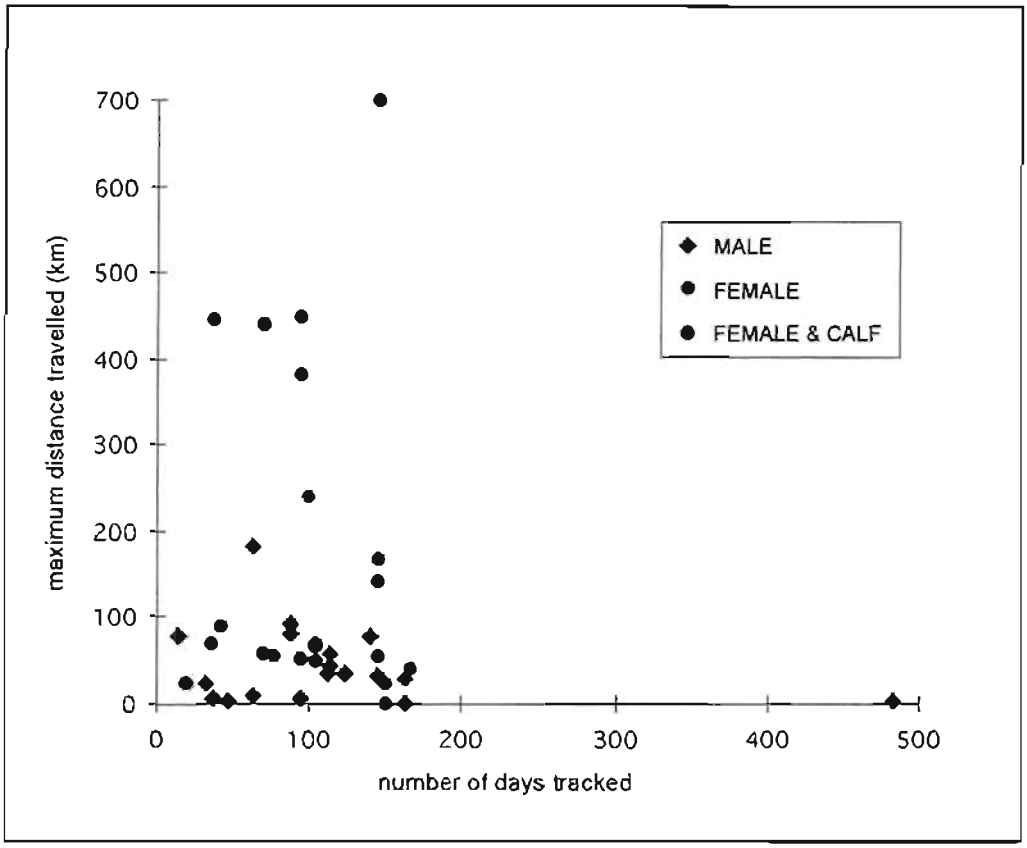


Figure 2.2: Movements of individual dugongs using conventional (radio) and satellite telemetry. The relationship between period tracked (days) and the span of coastline visited (km). This scatterplot shows individual dugongs tracked from Cleveland Bay (Townsville) and Starcke River (Marsh & Rathbun, 1990), and dugongs tracked from the Gulf of Carpentaria, Hinchinbrook, Shoalwater Bay and Hervey Bay (A.R. Preen, personal communication). The outlier (male dugong tracked for approximately 480 days) was the only dugong that was VHF-tracked, and for this reason, was unable to be tracked continuously.

levels. They can be used: 1) to identify individuals or family structure, 2) as a signature for identifying species, and 3) to reveal phylogenetic relationships among taxa. As genetic markers are inherited, they are permanent, and can provide information about historical population structure (e.g., if there has been a founder or bottleneck event) and the status of the gene pool (e.g., if the population is inbred).

Genetic markers are becoming increasingly useful tools for providing information for the protection and conservation management of endangered species (Baker *et al.*, 1994; Baker & Palumbi, 1994; Szabo, 1994). Information obtained from satellite tracking and other non genetic approaches to examining dugong populations will assist the interpretation of the information obtained via the genetic approach. It is the marriage of non genetic and genetic methods that will provide the most accurate assessment of intraspecific genetic structure for the dugong required for effective management.

There are two major considerations in the selection of a genetic marker to recognise intraspecific units: 1) the quality and type of sample, and 2) the level of discrimination provided by the particular marker. Allozymes, single-copy nuclear DNA (i.e., coding), satellite DNA (i.e., repetitive) and mitochondrial DNA (mtDNA), are all examples of regularly employed genetic markers for recognising intraspecific units. The highest level of resolution can be obtained from DNA sequences (introduced further in section 4.4), which in addition to providing information for a population level study, reveals patterns of genetic diversity and mutation rates.

2.2.3 Sample collection and storage requirements

Prior to the introduction of the Polymerase Chain Reaction (PCR), genetic studies were restricted to DNA samples that were of large quantity, high quality, and high molecular weight. Protein electrophoresis can be an economic and effective approach to examine the genetic population structure for the dugong. The main drawback of this method is the requirement for tissue that is of high quality (not decomposed), and is either fresh or frozen at -70°C.

PCR (detailed in section 4.3) enables DNA that is degraded or in small volumes to be utilised as a molecular tool. Laboratory technicians can avoid

the tedium of extracting clean, high molecular weight DNA, as several DNA extraction procedures which are rapid and omit some of the cleaning steps, have been developed (Langridge *et al.*, 1991; Walsh *et al.*, 1991). Some PCRs are performed directly on the tissue without any extraction procedure at all (e.g., McCusker *et al.*, 1992). In addition, PCR allows DNA to be preserved in a variety of ways, relieving the field worker of the burden of carrying liquid nitrogen or dry ice to freeze samples. In this study, many of the dugong samples were obtained from remote regions. Freezing tissue was not an option (nor allozymes as a molecular marker).

Not much tissue is required for the PCR. Olson *et al.* (1991), used PCR and DNA sequencing (sections 4.3 and 4.4 respectively) to distinguish planktonic larvae of sea cucumber species. The larvae are so similar that they cannot be identified on morphology alone. The PCR enabled the DNA from a single larva to be amplified, sequenced and identified. The broad spectrum of sources from which it is possible to amplify DNA, include decomposed tissues. It is possible to isolate mtDNA from samples that have been preserved and stored without future genetic analyses in mind such as formalin fixed, paraffin embedded tissues (Goelz *et al.*, 1985; Dubeau *et al.*, 1986; Impraim *et al.*, 1987; Shibata *et al.*, 1988; Greer *et al.*, 1991; Shiowaza *et al.*, 1992; Stein & Raoult, 1992) and museum specimens. It is therefore possible to examine historical genetic characteristics and allele frequencies (e.g., Diamond, 1990). DNA has been amplified from 100 year old feathers (Ellegren, 1991), ancient human bones (Hagelburg *et al.*, 1991a; Hagelburg & Clegg, 1993), an 1850 year old Egyptian mummy (Roewer *et al.*, 1991) and a 7000 year old human brain (Pääbo *et al.*, 1988). Woodward *et al.* (1994) reported DNA amplified from the bone fragments of a Cretaceous dinosaur (80 Myr old). However, the findings by Woodward *et al.* (1994) were challenged by a collection of researchers (Allan *et al.*, 1995; Hedges & Schweitzer, 1995; Henikoff, 1995; Zischler *et al.*, 1995) who claimed the DNA sequence actually represented modern human DNA contamination.

2.2.4 Protein electrophoresis

Protein electrophoresis (i.e., of isoenzymes and allozymes) was developed in the 1960's, and represents the first molecular approach widely applied to investigate population differentiation. Allozymes are specified by alleles, which are alternative forms of a gene at a locus. Therefore, by measuring allozyme variation in a population, a measure of genetic diversity is

obtained. The interpretation of allozyme data is based on a well developed body of theory, following Mendelian principles (i.e., the segregation of alleles and independent assortment of genes (Lawrence, 1989)).

2.2.5 Satellite DNA: minisatellites and microsatellites

Satellite DNAs are repeated units of sequence that are regarded as non-coding or 'junk DNA'. The repeating unit usually varies in length from two to several thousand base pairs. Depending on the length of the repeating unit, satellite DNA can be specified as either minisatellites (repeats of DNA sequence greater than a six base motif) usually located at centromeric and telomeric regions of a chromosome, or microsatellites (segments of DNA with very short sequence motifs (two to six bases), repeated in tandem), which are more evenly distributed throughout the genome (Queller *et al.*, 1993).

Satellite DNA families are considered to be inherited in a Mendelian manner. However, the DNA repeat units within a satellite locus behave in a non-Mendelian fashion. The repeat units frequently change in number, and spread from one repeating array to another throughout the satellite family by molecular processes of stochastic gain and loss, rather than accumulating independently within each length array (Amos & Dover, 1991). After several generations, a range of particular length variants produced by either the Mendelian or non-Mendelian mechanism, accumulate and characterise reproductively isolated populations. Such 'hypervariable' sequences have great potential to identify divisions between natural populations (Amos & Dover, 1991; Hoelzel & Dover, 1988).

Minisatellites are detected by excising genomic DNA with restriction enzymes (that do not cleave within the tandem repeats), followed by electrophoretic separation and transfer hybridisation (Southern blotting). This procedure permits size classes of alleles to be identified, usually in the size range of 1000 to 20,000 base pairs (Burke *et al.*, 1996; Hillis *et al.*, 1996). Two strategies are practised in the application of minisatellites. Using multi-locus minisatellites as markers to reveal variation, involves probing for a large number of hypervariable minisatellite loci. This approach was called 'DNA fingerprinting' by Jeffreys *et al.* (1985b) because the complex multifragment patterns obtained are usually unique to individuals. Multi-locus minisatellites or DNA fingerprints are powerful tools to test parentage

(Hillis *et al.*, 1996). The complicated banding pattern sometimes excludes multi-locus minisatellites as a favourable class of molecular marker because of problems with their interpretation; distinguishing heterozygotes from homozygotes is difficult when alleles are similar in size, or an excess of homozygosity is estimated because of undetected small fragments (Queller *et al.*, 1993). The alternative approach is single-locus minisatellites, where hypervariable loci are located one at a time by screening a genome library using specific probes at high stringency (Nakamura *et al.*, 1987). The economy and resolution of using single-locus minisatellites are improved by amplifying the DNA fragment of interest by PCR, followed by Restriction Fragment Length Polymorphic (RFLP) analysis (Jeffreys *et al.*, 1991). Single-locus minisatellites are also called variable number tandem repeats (VNTRs).

Microsatellites are detected using the method of the PCR (section 4.3), and acrylamide gel electrophoresis to separate a profile of individual or population specific bands (Burke *et al.*, 1996). Microsatellite loci are numerous in eukaryotes (Tautz & Renz, 1984) and distributed throughout the genome (Weissenbach *et al.*, 1992). In humans, there are at least 35,000 $(CA)_n$ repeat loci alone (Weber, 1990). Three and four-base repeats are rarer, but their numbers are still in the thousands (Edwards *et al.*, 1991). Repeat motifs are confusing to the DNA replication process, with length variations (alleles) arising because the DNA polymerase occasionally 'slips'. The resulting alleles differ in the number of repeats and are inherited in a Mendelian fashion. Microsatellite loci that have ten or more repeat motifs make excellent genetic population markers as they tend to have multiple alleles (Weber & May, 1989; Weissenbach *et al.*, 1992). Microsatellites tend to be favoured over minisatellites as molecular markers, as the latter are sometimes clustered in particular regions (Royle *et al.*, 1988) which may reduce their informativeness. In addition, microsatellites are easier to resolve than minisatellites. For example, as VNTR loci are usually a part of large restriction fragments (several kilo bases long), the difference of one repeat unit (on such a large fragment) is not large enough to differentiate their migration on a gel.

Because mtDNA is maternally inherited (section 2.2.10), microsatellites were explored as a nuclear marker to examine bi-parental genetic patterns within and among dugong populations. The application of microsatellites is further introduced and discussed in Chapter 9.

2.2.6 Single-copy nuclear DNA

Restriction analysis of single-copy nuclear (scn) DNA is detailed by Avise (1994). Traditionally the method relied on transfer hybridisation (Southern, 1975), using scnDNA sequences cloned into a biological vector as a probe. A probe may be composed of a gene(s) of known function which is derived from complementary (c) DNA (produced by reverse transcription of a specific messenger ribonucleic acid). Alternatively, the probe may be composed of anonymous tracts of DNA drawn at random from a genome library (Karl & Avise, 1993). Anonymous single-copy probes may include regions flanking the gene which are often junk DNA (non coding stretches of DNA which intersperse coding DNA, e.g., introns). As non coding sequences are usually not under the constraints of selection, they tend to be highly variable, and are therefore suitable as genetic markers for intraspecific studies (e.g., Karl & Avise, 1992; Karl *et al.*, 1992). Interpreting scnDNA RFLP data is comparable to the interpretation of allozymes. Restriction analysis is superior to allozymes as it can exploit a more extensive pool of genetic variation for intraspecific studies. Karl *et al.* (1992) used RFLP analysis of anonymous nuclear loci to ascertain the global genetic population structure of the green turtle (*Chelonia mydas*). They found moderate male-mediated gene flow between natal sites, contrasting with highly structured female (mtDNA) lineages.

The most challenging aspect of scnDNA RFLP analysis, is the construction of suitable probes, hence the major drawback in employing scnDNA analyses (in comparison to allozymes) is the expense in labour and materials (Avise, 1994).

2.2.7 Random amplified polymorphic DNA (RAPDs)

Random amplified polymorphic DNA (RAPDs) is a method in which arbitrary oligonucleotides (primers) of about ten bases are annealed to template (genomic) DNA, and products are obtained via the PCR (section 4.3). The PCR products are electrophoresed through agarose and stained to produce a pattern of bands. Bands present in one individual may not be present in another for a variety of reasons, chiefly variation in the primer annealing sites (Welsh & McClelland, 1990; Williams *et al.*, 1990). Reproducibility is the main problem with using RAPDs. The benefit of using RAPDs is that only small amounts of tissue are required. The RAPD approach appears to be popular as a marker in studies of insect species

(Black, 1993; Haymer, 1994), but has not yet been employed widely in mammalian population biology (Avise, 1994).

2.2.8 Approaches based on DNA sequence data

Information gained from sequencing is absolute, providing the highest resolution of genetic data that can be obtained. Sequencing yields up to ten times more data compared to restriction enzyme site/fragment data (Geffen *et al.*, 1992). The data can be kept for future reference (permanent) and enables direct comparison between different studies. Almost all population studies based on DNA sequence data now use PCR (section 4.3) to amplify the region of interest. PCR products can be sequenced directly (Wrischnik *et al.*, 1987), an approach further simplified by quick methods of PCR product purification (e.g., Werle *et al.*, 1994) and a number of DNA sequencing commercial kits (section 4.4).

2.2.9 Genetic markers: Nuclear verses Mitochondrial DNA

Eukaryotic cells contain both nuclear and organelle (e.g., mitochondrial) genomes. Genetic approaches such as fingerprinting and microsatellite analysis target the DNA of the nuclear genome as it contains almost all of the cell's genetic material. Although some satellite sequences are known from the mitochondrial genome, the amount of satellite DNA is minimal compared to the nuclear genome. The properties of mtDNA are such that many population studies involving DNA sequences and RFLP analysis target this genome.

2.2.10 Mitochondrial DNA

Most population studies target the mitochondrial genome because of its unique properties. In vertebrates, mitochondrial DNA (mtDNA) evolves five to ten times more rapidly than nuclear DNA (Brown *et al.*, 1979; Brown *et al.*, 1982) with base replacement rates in various animals ranging between 0.1% to 2% per million years (discussed further in Chapter 8). The extensive range of polymorphism of mtDNA, makes it an ideal genetic marker to differentiate populations, phylogeographic units (groups of populations), closely related subspecies and species (Lansman *et al.*, 1983; Avise, 1986; Ashley & Wills, 1987; Plante *et al.*, 1989). As mtDNA is maternally inherited, it is not complicated by the effects of sexual recombination (Avise *et al.*, 1987). Paternal leakage in mtDNA inheritance has been known to

occur with low frequency (Gyllensten *et al.*, 1985; Avise & Vrijenhoek, 1987), but not to a level that has been known to complicate interpretation.

Mitochondrial DNA does not undergo sexual recombination; it is clonally inherited. For this reason homoplasmy is expected, and indeed, site homoplasmy is observed to be common for most mammals (Potter *et al.*, 1975; Hayashi *et al.*, 1978; Bendall & Sykes, 1995). However, length heteroplasmy (where DNA molecules are not identical in length within an individual) is being discovered to be much more common than originally believed (Buroker *et al.*, 1990; Ghivizzani *et al.*, 1993; Casane *et al.*, 1994; Hoelzel *et al.*, 1994; Xu & Arnason, 1994; Bendall & Sykes, 1995). Site heteroplasmy (where particular sites/bases of the DNA molecule vary within an individual) is not reported as commonly as length heteroplasmy, although it has been known to occur in humans (Comas *et al.*, 1995). If heteroplasmy were extensive, it may complicate mtDNA studies. For this reason, I checked for heteroplasmy within the chosen gene region as indicated in Chapter 10. Heteroplasmy is common in certain species (Solignac *et al.*, 1983). However, because these cases are unusual, they are presumed to have little impact on the routine survey of animal mtDNA (Avise *et al.*, 1987).

Variable and conserved regions have been identified in mtDNA for a broad range of organisms via comparative sequencing and restriction enzyme mapping (Upholt & Dawid, 1977; Brown *et al.*, 1982; Cann *et al.*, 1984; Solignac *et al.*, 1986). The more varied (i.e., less conserved) gene regions are suitable for recognising intraspecific units and investigating population structure. Regions that are more conserved, such as those that code for ribosomal RNA (rRNA), have very low levels of redundancy and are unlikely to retain mutations (Brown & Simpson, 1981; Ferris *et al.*, 1981; Cann & Wilson, 1983; Cann *et al.*, 1984). These conserved regions are shared between some of the most distantly related organisms. As a consequence, conserved regions are appropriate for comparisons at higher taxonomic levels.

The cytochrome *b* gene (protein coding region of mtDNA) is known to be suitable as a population marker, and was initially investigated to verify if it was adequately variable for this study (Chapter 5). A more variable segment of mtDNA than the cytochrome *b* gene is the control region or D-loop, which is recognised as a 'hot spot' for base substitution both within

(Aquadro & Greenberg, 1983; Fauron & Wolstenholme, 1980) and between (Fauron & Wolstenholme, 1980) species. Within the control region there are two hypervariable segments of DNA (the ends) which are targeted for their particularly high rate of mutation (Wakeley, 1993). The hypervariable region at the 5' end of the control region was chosen in preference to the cytochrome *b* gene region of mtDNA to resolve intraspecific units for the dugong (Chapter 7 & 8) as it proved to be more suitable (i.e., more variable).

2.3 PATTERNS IN MARINE MAMMALS: GENETIC STRUCTURE and DIVERSITY

2.3.1 Measures of genetic diversity

Heterozygosity and polymorphism were first introduced as measures of genetic diversity using data from protein electrophoresis (section 2.2.4). **Heterozygosity** (h) is the mean percentage of individuals that are heterozygous per locus (the observed heterozygosity, H_O) (Avise, 1994). A measure of 0.5 (frequency) for an allele in a population is generally considered as 'high' heterozygosity. For any given locus, h is the probability that any two alleles chosen randomly from a population will be different from each other. In a randomly mating population, h is also the expected heterozygosity (H_e). That is, the heterozygosity predicted from allele frequencies (Li, 1997). **Polymorphism** is the persistence of multiple alleles at a gene locus. A locus is polymorphic if the most common homozygote (i.e., allele) occurs at a frequency of less than 90% in the population. Polymorphism may be measured either by the number of polymorphic sequences in the sample, and/or the number of polymorphic sites. Both measures are highly dependent on sample size (Nei, 1987).

For mtDNA, an equivalent statistic to estimate genetic diversity (i.e., heterozygosity) at the nucleotide data level (where a locus equates with a specific stretch of DNA sequence) is **nucleotide diversity** (π) (Nei & Li, 1979; Nei & Tajima, 1981; Nei, 1987; Nei & Jin, 1989). There are two different ways to define nucleotide diversity: 1) the average of the proportion of different nucleotides (substitutions) between two sequences (p_{ij}) over all pairwise comparisons of haplotypes (specific DNA sequences), and 2) the average of the number of nucleotide substitutions per site between two haplotypes (d_{ij}). The total variance of nucleotide diversity d_{ij}, can be estimated from p_{ij} (Nei

& Jin, 1989). Depending on the species and loci studied, within-population nucleotide diversities can fall in the range of 0.005 to 0.020 (0.5 to 2%) as observed for species of *Drosophila* (Charlesworth & Langley, 1989).

Haplotype diversity (h) is a DNA sequence analogue to heterozygosity used in the study of protein polymorphism (Nei & Tajima, 1981; Tajima, 1983). Its calculation does not require assessment of the magnitude of genetic divergence between haplotypes (Avise, 1994), and it is defined as the probability that two haplotypes randomly chosen from a population are different (Nei & Tajima, 1981). Nucleotide diversity is more informative than haplotype diversity, since the former gives information on the extent of DNA difference between two randomly chosen genes, whereas the latter gives information only on whether a pair of haplotypes are the same or not (Tajima, 1983). For rapidly evolving genomes such as mtDNA, haplotype diversity sometimes approaches 1.0 (Avise *et al.*, 1989; Avise, 1994).

Two other quantities for measuring genetic variation are the number of haplotypes (Ewens, 1972) and the number of polymorphic nucleotide sites (Watterson, 1975). However, these measures are strongly dependent on sample size, and for these reasons Tajima (1983) considers nucleotide diversity (i.e., average number of nucleotide differences) to be the most accurate measure of within-population genetic variation. Both nucleotide and haplotype diversity are accompanied by a large variance attributed to: natural selection, recent migration between populations, and stochastic error generated by random genetic drift (Tajima, 1983).

2.3.2 Models of population structure

Aspects of population structure include age and sex composition, and the extent of genetic subdivision (Wright, 1951). The determination of which genetic model best approximates the population structure, is one of the most fundamental pieces of information for a species requiring management (Hillis & Moritz, 1990). A population that is large, interbreeds randomly, and has a lack of genetic subdivision, is known as a panmictic population (Wright, 1951). The absence of genetic subdivision (i.e., dugongs occurring as one panmictic population) is the null hypothesis of this study.

The isolation-by-distance model (Wright, 1943; 1951; Slatkin, 1993) is based on a correlation between geographic and genetic distance (Hartl & Clark,

1989). It represents an intermediate degree of 'inbreeding' where only adjacent populations exchange genetic information (Wright, 1951). Assuming roughly constant rates of migration, geographic and genetic distances are positively correlated for individuals within a species (Hartl & Clark, 1989).

Stepping-stone models differ from Wright's isolation-by-distance models in that populations are organised discretely (Hartl & Clark, 1989), and assume that only migration into adjacent populations is allowed.

Population subdivision entails an 'inbreeding-like effect' in terms of excess homozygosity (Hartl & Clark, 1989). This effect can be measured in terms of the decrease of the proportion of heterozygote genotypes. A subdivided population has three levels of heterozygosity: heterozygosity of an individual (I) in a subpopulation, the expected heterozygosity of an individual in an equivalent random mating subpopulation (S), and the expected heterozygosity of an individual in an equivalent random mating total population (T) (Hartl & Clark, 1989). These measures of heterozygosity relate to Wright's (1951) *F*–statistics: the inbreeding coefficient F_{IS}, represents the reduction of heterozygosity of an individual due to non random mating within its subpopulation; the fixation index F_{ST}, is the reduction of heterozygosity in a subpopulation due to random genetic drift; and F_{IT} is a measure of the reduction in heterozygosity of an individual relative to the total population.

2.3.3 Range size, range type and genetic variation

'The idea that types and levels of genetic variation in populations can be related to temporal and spatial patterns of environmental variation has been a persistent theme in evolutionary biology' (Selander & Kaufman, 1973). Heterogenous environments are expected to be positively correlated with genetic diversity on theoretical grounds (e.g., Maynard Smith, 1970; Sammeta & Levins, 1970). This has been demonstrated in *Drosophila* via laboratory breeding experiments (Powell, 1971; McDonald & Ayala, 1974). In addition to a heterogenous environment, other features recognised for promoting the survival of a lineage (hence genetic diversity), are a wide geographic range, high population density and large population size (Diamond, 1990).

2.3.4 Marine mammal genetic diversity

In the search to relate genetic patterns to environmental factors, the notion has arisen that marine mammals are characterised by low genetic diversity. This notion is not entirely substantiated by the literature. Low genetic diversity of marine mammals is advocated in review papers by Nevo (1978) and Nevo *et al.* (1984). Nevo (1978) included two species of pinnipeds (seals) (*Mirounga angustirostris* and *M. leonina*) in his review of allozymic variation from a total of 243 species; *M. angustirostris* had a heterozygosity and polymorphism level of 'zero'. The Nevo *et al.* (1984) review of mammalian allozyme studies is somewhat more comprehensive. Genetic diversity measures of six pinnipeds (*Cystophora cristata, Eumetopias jubatus, M. angustirostris, M. leonina, Odobenus rosmarus, Pagophilus groenlandicus*) and one cetacean (*Balaenoptera acutorostrata*) were included. The appropriate conclusion from the reviews by Nevo (1978) and Nevo *et al.* (1984) is that 'pinnipeds are characterised by low genetic diversity', not that marine mammals in general are characterised by low genetic diversity as has been asserted.

Nonetheless, there are valid reports of other marine mammal species that do conform to the generalisation of low genetic diversity. Low within-species genetic variation appears to be prevalent in a number of cetaceans. For example, low within-species genetic diversity was observed in the control region of minke and killer whales (*B. acutorostrata* and *Orcinus orca* respectively) and Commerson's dolphin (*Cephalorhynchus commersonii*) (Hoelzel *et al.*, 1991). Table 2.2 summarises findings of allozyme research on marine mammals. It indicates that, with the exception of pinnipeds, there are many marine mammal species, including the Florida manatee (McClenaghan & O'Shea, 1988), with heterozygosities comparable to the mammal average.

Amos (1996) cautioned against connecting low genetic variability of cetaceans with past exploitation (hunting) as few, if any cetacean populations were hunted 'low enough for long enough' for the effect to be detected by nuclear markers. Conversely, Amos (1996) cautions against management complacency with exploited species that appear to have retained 'healthy' levels of genetic variability. Mitochondrial DNA has a greater sensitivity for detecting recent population changes because of a

higher rate of mutation in comparison to nuclear protein coding loci (Brown *et al.*, 1979; Brown *et al.*, 1982). However, Amos (1996) did not extend the computer simulations to test whether mtDNA markers had an ability to detect changes in genetic variability associated with recent (i.e., 200 years) reductions in population size.

Table 2.2: Allozyme heterozygosity and polymorphism observed for terrestrial and aquatic animals.

Species	*Heterozygosity*	*Polymorphism (no. of loci)*	*Reference*
Terrestrial vertebrates	0.041	0.180	Nevo (1978)
"	0.069	-	Nevo *et al.* (1984)
Marine vertebrates	0.061	0.203	Nevo (1978)
"	0.046	-	Nevo *et al.* (1984)
Mammalian average	0.051	0.206	Ayala (1982)
Marine mammals	0.011	0.148	Nevo (1978)
"	0.018	0.118	Nevo *et al.* (1984)
COASTAL/INSHORE			
Pinniped average	0.019	-	Testa (1986)
Florida manatee (*Trichechus manatus latirostris*)	**0.050**	**0.300**	**McClenaghan & O'Shea (1988)**
PELAGIC			
Cetacean average	0.043	-	as listed below
Sei whale (*Balaenoptera borealis*)	0.023	0.180	Wada & Numachi (1991)
Sei whale (Iceland)	0.047	0.175	Daníeldóttir *et al.* (1991)
Sei whale (North Pacific & Ant.arctica)	0.021	0.158	Wada (1988)
Fin whale (*B. physalus*)	0.018	0.163	Wada & Numachi (1991)
Fin whale (Iceland)	0.071	0.275	Danieldóttir *et al.* (1991)
Fin whale (Spain)	0.089	"	"
Bryde's whale (*B. edeni*)	0.020	0.102	Wada & Numachi (1991)
Minke whale (*B. acutorostrata*)	0.053	0.216	"
Minke whale (Japanese)	0.023	0.070	Wada (1983)
Minke whale (North Atlantic)	0.046	0.095	Simonsen *et al.* (1983)[1]
Dall's porpoise (*Phocoenoides dalli*)	0.058	-	Winans & Jones (1988)

[1]Included in the average calculated by Nevo *et al.* (1984) for marine mammals.

2.4 REVIEW OF DUGONG HISTORY AND BIOLOGY RELEVANT TO THIS STUDY

2.4.1 Historical ecology of Sirenia

The mammalian Order Sirenia is characterised by low species richness. Even at their peak diversity during the Miocene (figure 2.3), there were only about a dozen, mostly monotypic, genera worldwide (Domning, 1978). The most influential factor in the distribution and evolutionary success of sirenians has been the quality and/or abundance of their aquatic food plants, and their ability to adapt to changes in food type. Seagrasses are believed to have evolved in the late Cretaceous (den Hartog, 1970; Specht, 1981), pre-dating the appearance of sirenians in the early Eocene (Domning, 1981). Eocene seagrasses are known from the Tethyian and Indian Oceans (Domning, 1981). Sirenian food availability has probably been directly influenced by competition with other herbivores, change of climate, and geography (Domning, 1982).

The extant members of the Order Sirenia (figure 2.4) are the dugong, *Dugong dugon* (Müller, 1776) and three species in the Family Trichechidae (manatees). According to morphological (Domning *et al.*, 1986; Novacek *et al*, 1988; Novacek, 1990) and molecular (de Jong *et al.*, 1981; Rainey *et al.*, 1984; Barriel *et al.*, 1993; Springer & Kirsch, 1993; Irwin & Arnason, 1994; Lavergne *et al.*, 1996; Porter *et al.*, 1996; Stanhope *et al.*, 1996; Ozawa *et al.*, 1997; Springer *et al.*, 1997) data, the orders most closely related to Sirenia are the land dwelling subungulates, including the Orders Proboscidea (elephants), Hyracoidea (hyraxes) and Tubulidentata (aardvarks). However, sirenians have often been confused with other marine mammals of the more distantly related Orders Carnivora and Cetacea because of similarities in body shape, adaptations and habitat. In the 19th Century the walrus (*Odobenus rosmarus*) was placed in the genus *Trichechus* with manatees because of their striking external resemblance (see Reynolds & Odell (1991) for reproduced lithographs). Today, dugongs are sometimes confused with the finless porpoise (*Neophocaena phocaenoides*) (e.g., see Jones, 1981), although the single blow-hole of the porpoise compared to the double nostrils of the dugong distinguishes them easily (Jefferson *et al.*, 1993). In Townsville, Australia (19°15'S, 146°45'E), beached carcasses of the Irrawaddy

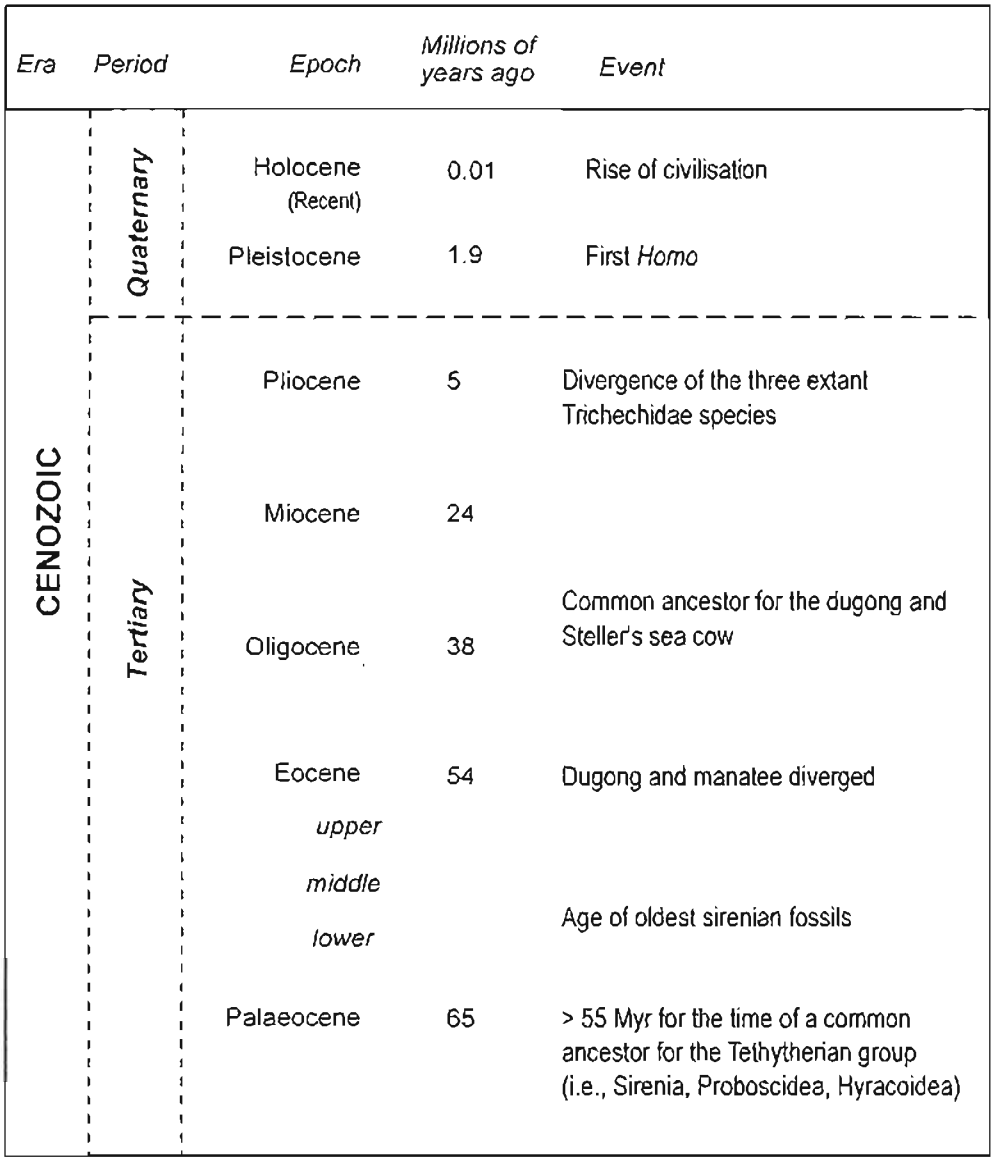


Figure 2.3: Sirenian events mapped onto a Cenozoic geological time scale (adapted from Keeton, 1980). The divergence between the extant Trichechidae species is believed to have occurred sometime during the Pliocene (Domning, 1982). The ancestors of the Steller's sea cow and the dugong apparently split during the Oligocene. The divergence time between the dugong and Florida manatee is debatable with fossil evidence estimating a divergence of 30 to 40 Myr ago (Domning & Hayek, 1986), and molecular data ranging in estimates between 17 and 45 Myr ago (Rainey *et al.*, 1984; Lavergne *et al.*, 1996). Seagrasses appeared in the late Cretaceous, pre-dating the lower Eocene appearance of sirenians (Domning, 1981; 1982).

river dolphin (*Orcaella brevirostris*) are often mistakenly reported as dugongs by members of the public (personal observation).

Sirenian ancestors are believed to have evolved during the Palaeocene (figure 2.3) in the shallow coastal waters of the Tethyian seaway (Savage & Russell, 1983). Hence the term 'tethytherian' ('phenacodontoid') is used sometimes to describe some members of the subungulate group (McKenna, 1975; Savage & Russell, 1983). Primitive sirenians spread from the Tethyian seaway into new regions including the New World (American) coastline. The oldest and most significant sirenian fossil is a skull of *Prorastomus sirenoides* Owen, 1855, found in Jamaica, which is dated to the lower or middle Eocene (Domning, 1982; Donovan *et al.*, 1990; Savage *et al.*, 1994) (refer to figure 2.3 for a geological time scale).

Most fossil sirenians and taxa belong to the family Dugongidae which was widespread by the upper Eocene, reaching its peak diversity (relative to other sirenians) in the Miocene (Domning, 1982). Dugongid fossils have been found in the Mediterranean (Spain to Egypt), western Europe, southeast of North America, the Caribbean, Indian Ocean, South America and North Pacific (Domning, 1982). The fossil evidence suggests that early trichechids were much less abundant and diverse than dugongids, and the Tertiary fossils referred to the Family Trichechidae originate only from the New World (mainly South America) (Domning, 1982).

Fossil trichechids and dugongids are not found together outside South America (Simpson, 1932; Domning, 1982). Dugongids were present in North America and the West Indies throughout most of the Tertiary. However, trichechids did not appear in South America until the Miocene, or in North America until the Pleistocene. At around the same time, dugongids became extinct from the western Atlantic (as well as from Europe and the Mediterranean). It appears that trichechids took over the New World from the dugongids, either by displacement or post-extinction replacement (Domning, 1982).

The Trichechidae appear to have been isolated in South America since at least the mid-Tertiary. By the Pliocene, trichechids isolated in the Amazon basin had evolved into the Amazonian manatee (*Trichechus inunguis*, figure 2.5). Trichechids present in the Caribbean during the time of divergence from the Amazonian manatee, are represented by the West

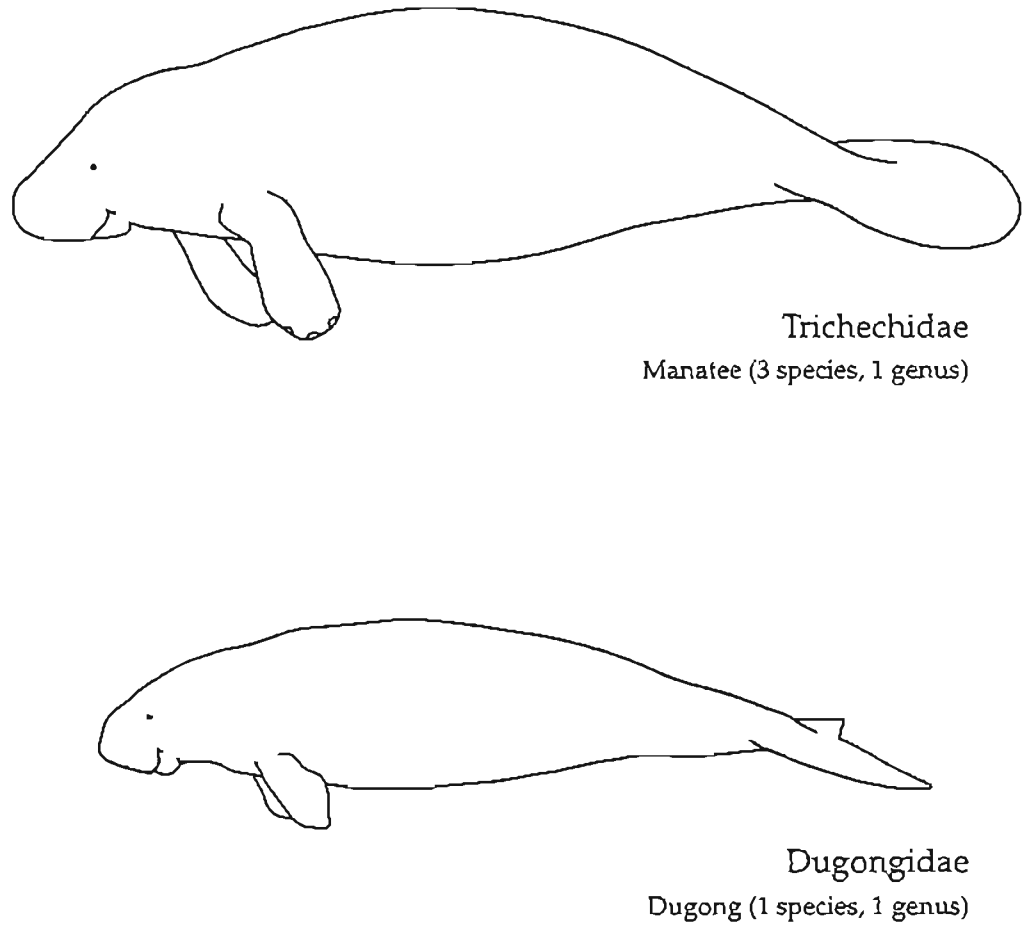


Figure 2.4: Extant sirenian families. The tail of the dugong, with its whale-like flukes and median notch, distinguishes it immediately from the manatee. Dugongs are more streamlined and cetacean-like than manatees. Manatees spend most of their lives in fresh or brackish water whereas the dugong is strictly marine (refer to section 2.4.2 for exceptions). This figure was reproduced from Jefferson *et al.* (1993).

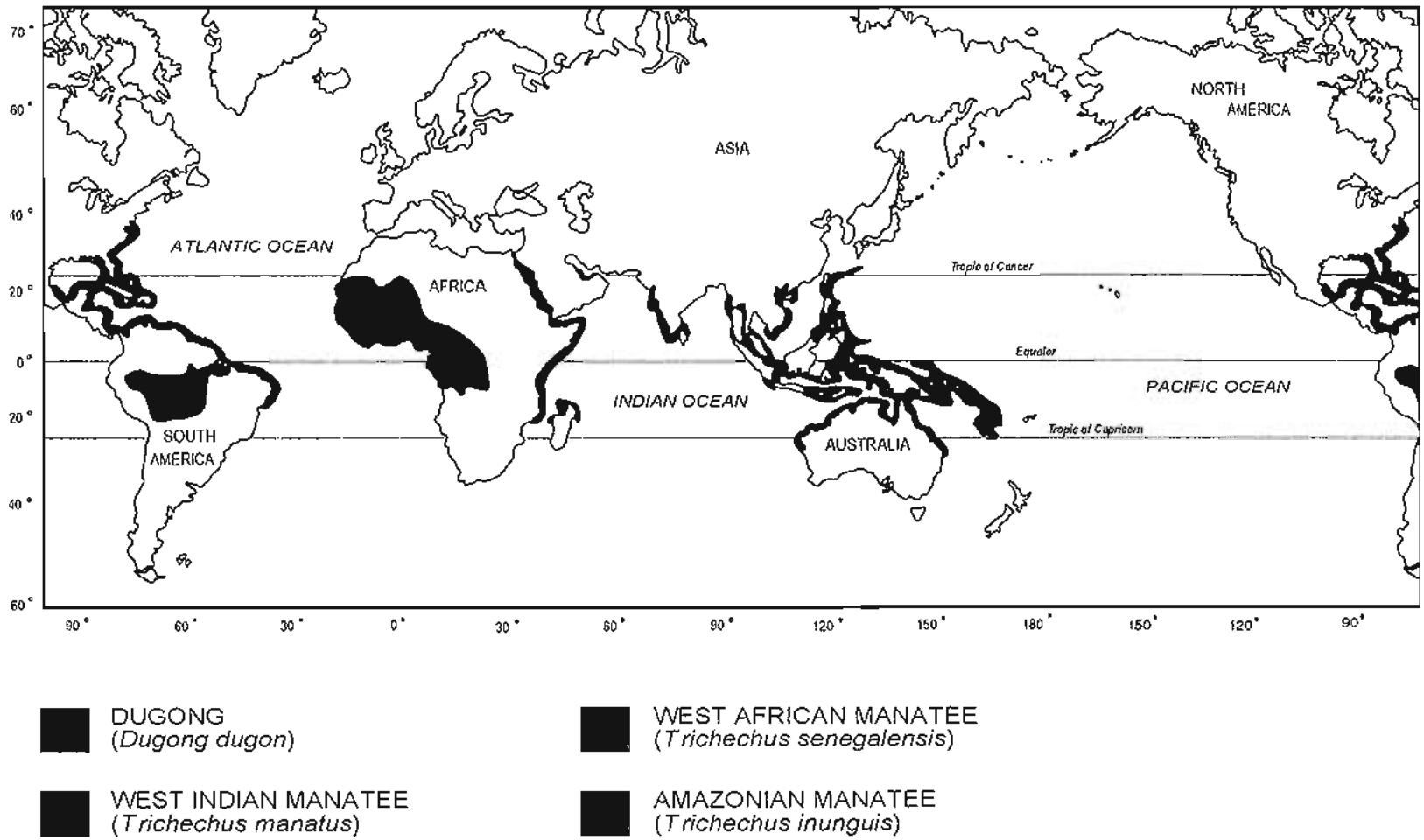


Figure 2.5: Estimated ranges of extant sirenians (taken from Reynolds & Odell, 1991).

Indian manatee (*T. manatus*). The West African manatee (*T. senegalensis*, figure 2.5) is believed to have arrived at its present day habitat by chance colonisation by trichechids from the Caribbean sometime during the Pliocene (Domning, 1982).

Members of the family Dugongidae were present in the North Pacific from early Miocene to Recent (Domning & Furusawa, 1995). Species of *Metaxytherium* are considered to have given rise to Steller's sea cow and are believed to have dispersed to the Pacific through the Central American Seaway (Domning & Furusawa, 1995). Steller's sea cow was the only sirenian that adapted to and inhabited cold temperate to subarctic waters. The dugong and Steller's sea cow diverged from a common ancestor around the Oligocene (figure 2.3; Savage, 1976; Domning, 1994).

The Recent dugong probably entered the Pacific from the Indian Ocean (Domning, 1994; Domning & Furusawa, 1995). Despite its vast range (following section; figure 2.5), there is no fossil record of the dugong other than a few fragments of around Pleistocene age from Australia and Papua New Guinea (Domning & Furusawa, 1995). These include: dugong subfossils from New South Wales, south of their present Australian range on the east coast (Etheridge *et al.*, 1897; Etheridge, 1905) which are estimated to date from at least 100,000 years before present (i.e., the last high sea level phase); vertebrae and a skull fragment, probably Pleistocene, from Woodlark Island, Papua New Guinea (fossil catalogue number, AM F5795; Etheridge, 1900; deVis, 1905; Molnar, 1982); a rib and vertebral fragments (Miocene) from the Darai Limestone, Papua New Guinea (R. Wells, personal communication); and an 'unconvincing report' (D. Domning, personal communication) of a fragment of a dugongid mandible from the lower Pliocene Loxton sands of South Australia, recorded by Pledge (1992).

2.4.2 Dugong distribution and movements

The dugong has a longitudinal range between 170°E in the New Hebrides and 30°E at the head of the Gulf of Suez, and a latitudinal range between 30°N and 30°S (Bertram, 1981). Its distribution is discontinuous from southeast Africa, the Red Sea, the Persian Gulf, western coast of India to Sri Lanka (Jones, 1981), throughout Indonesia (Hendrokusomo & Tas'an, 1981) and the Pacific islands (Brownell *et al.*, 1981), the Ryukyu islands and the northern coast of Australia (Nishiwaki *et al.*, 1981).

The evolution and historical distribution of the dugong have been primarily influenced by their potential to adapt to food type and availability respectively (Domning, 1981; 1982). These factors continue to influence the dugong's range today. The mainly coastal range of radiotracked dugongs overlaps with their seagrass food (Marsh & Rathbun, 1990). Dugongs have been reported to move out of local areas of low temperature (less than 20°C at the high latitudinal limits of their range) (Anderson, 1986; 1994; Preen, 1993; Marsh *et al.*, 1994b). Thus the range of the dugong (figure 2.5) is primarily related to food availability, and either directly or indirectly affected by temperature, restricting them to coastal tropical and sub tropical waters of the Indo-Pacific.

Dugongs generally reside in bays, shallows and some offshore reefal areas (e.g., Ashmore Reef (12°15'S, 123°05'E), Whiting (in press)) with extensive beds of seagrass (Heinsohn *et al.*, 1977). They have been sighted up to 58 km offshore, in water estimated to be as deep as 37 m (Marsh & Saalfeld, 1989). The reason for dugongs venturing so far offshore is not entirely understood. However, seagrasses known to be eaten by dugongs occurred in the areas where they were sighted or tracked (Marsh & Rathbun, 1990). Although 'strictly marine', dugongs have been radiotracked 10 km up the tidal reaches of a creek (Marsh & Rathbun, 1990). Generally considered as rather slow swimmers, travelling at an average speed of 3 km/hour (Marsh & Rathbun, 1990), dugongs can put on a burst of speed over short distances when pursued reaching 20 km/hr (10 to 12 knots) (Marsh *et al.* 1981).

Dugongs are most frequently seen individually, or in very small groups (Preen, 1993). In some regions such as Moreton Bay, large and distinct herds (up to several hundred) are observed regularly, a phenomenon attributed to specialised feeding behaviour (Preen, 1993; 1995b). Despite their dispersal ability, there is no evidence to suggest that mass migrations of dugongs occur to breed (e.g., on a scale comparable with humpback whales (*Megaptera novaeangliae*) (Chittleborough, 1965)). Satellite tracking studies of dugongs have shown that some individuals can be relatively sedentary for months at a time, while others may move several hundred kilometres in a few weeks, see figure 2.2 (Marsh & Rathbun, 1990; Preen, 1993; A.R. Preen, personal communication). Even when accompanied by a calf, females appear to be moving greater distances than males (figure 2.2). Movements are usually along coastlines between feeding grounds and/or to areas where there are other dugongs, in a pattern which suggests that

dugongs navigate (A.R. Preen, personal communication). Although tracking studies show that dugongs are individualistic in their movements and have potential for dispersal, these studies have not identified intraspecific subdivision.

2.4.3 Seagrass specialist

The dugong is a seagrass specialist (Johnstone & Hudson, 1981; Marsh *et al.*, 1982; Lanyon *et al.*, 1989; Preen, 1995a; 1995b) with the digestive physiology of a nonruminant herbivore (Marsh *et al.*, 1977). However, seagrasses are not the only food eaten by dugongs. Dugongs will ingest brown algae in times of critical food shortage (Spain & Heinsohn, 1973; Heinsohn & Spain, 1974; Marsh *et al.*, 1982), even though they are apparently unable to digest algae as a food source efficiently (Marsh, 1981; Marsh *et al.*, 1982). Dugongs also consume benthic animals both incidentally and deliberately. Sea cucumbers were found in the gut contents of dugongs caught in shark nets off Townsville (19°16'S, 146°49'E). In Shark Bay (25°25'S, 113°35'E), dugongs have been seen digging for mussels and sea pens (Anderson, 1989). Preen (1995a) found that dugongs fed deliberately on ascidians in Moreton Bay (24°25'S, 153°20'E).

Deliberate omnivory appears to be limited to high latitudinal limits of the dugong's range and may be explained by the compounding effects of seasonal stresses such as cold water temperatures and limited nitrogen availability (lower seagrass abundance) (Preen, 1993). This dietary extension in times of environmental and nutritional stress is accompanied by regular local movements to warm water areas during the winter (Preen, 1993). The consumption of macro invertebrates (with seagrass) by dugongs that inhabit less stressful and more tropical regions, is believed to be incidental (Preen, 1995a).

Although dugongs appear to be facultative omnivores, their feeding grounds effectively remain restricted to seagrass meadows, as the benthic invertebrates on which they feed, live near, or in, seagrass. If dugong movements and distribution are influenced by food availability, then mapping seagrass distribution should provide an indicator of suitable dugong habitat. However, recognising 'dugong habitat' is not as straightforward as this, as only some seagrass meadows are favoured by dugongs (Preen, 1993).

Compared to their tropical neighbours, dugongs of Moreton and Shark Bays are believed to be under greater nutritional stress, and it is believed they form large herds for cultivation grazing and are obligate omnivorous in order to meet their nutritional requirements (Preen, 1995a; 1995b). If dugongs in peripheral populations differ in their behaviour, movements and distribution from their more central and tropical neighbours, a different genetic structure would not be unexpected for those peripheral populations.

2.4.4 Status and demography

The genetic uniqueness of a population is perhaps the strongest argument for its conservation, because of its importance to the species as a source of genetic diversity. If a population is also critically low in numbers, this increases the urgency of preservation efforts. Although much literature is available from incidental sightings of dugongs, aerial surveys are the only feasible method of estimating dugong numbers. Improvements in aerial survey methodology (Marsh & Sinclair, 1989) have resulted in a reassessment of dugong abundance (tables 2.3 & 2.4). In waters of Australia and the Torres Strait, dugongs have been extensively surveyed with an estimated total of about 85,000 (table 2.3; Marsh, 1996b). This is an underestimate as: 1) not all potential habitats have been surveyed, and 2) the correction factors for dugongs which are not seen in the turbid areas are very conservative. Aerial surveys provide a measurement of the impact of habitat loss, hunting, and netting on dugongs in Australia. For example, aerial surveys suggest that in the southern Great Barrier Reef between Dunk Island and Hervey Bay, the number of dugongs has fallen by about 50% over the past eight years, probably because of anthropogenic impacts and habitat loss (Preen & Marsh, 1995; Marsh *et al.*, 1996).

Only limited estimates are available for the sizes of dugong populations outside the waters of Australia and Torres Strait. Anecdotal evidence suggests that in most areas, densities are not nearly as high as in Australia (Bertram & Bertram, 1973; Nishiwaki *et al.*, 1979; Hendrokusumo & Tas'an, 1981; Jones, 1981; Nishiwaki & Marsh, 1985). An exception is the Arabian Gulf where dugong numbers are estimated to be 7307 (s.e. ±1302) based on aerial survey data (table 2.4, Preen, 1989b; Preen *et al.*, 1989). However, the level of anthropogenic impacts suggests that the future of the dugong in this area is uncertain. Outside of Australian and Torres Strait waters, an assessment of dugong numbers based mainly on anecdotal information

suggests that they are diminishing and in most of their range remain, 'rare and susceptible to extinction' (Bertram, 1981; Marsh & Lefebvre, 1994).

Table 2.3: The most recent estimates of dugong numbers in northern Australia. The Queensland coast of the Gulf of Carpentaria, and Western Australia's Kimberley coast have yet to be surveyed. These numbers are believed to be underestimates, due to the use of conservative correction factors to compensate for dugongs unseen because of water turbidity (H.D. Marsh, personal communication).

Region	*Number ± s.e.*	*Date of survey*	*Reference*
Shark Bay, Western Australia	10,529 ± 1,464	*July 1994*	Preen *et al.* (1997)
Exmouth Gulf & the Ningaloo Reef, Western Australia	1,974 ± 588	*July 1994*	Preen *et al.* (1997)
Northern coast, Northern Territory	13,800 ± 2,683	*December 1983*	Bayliss (1986); Bayliss & Freeland (1989)
Western Gulf of Carpentaria	16,846 ± 3,259	*February 1985*	Bayliss & Freeland (1989)
Wellesley Island area, Queensland	4,067 ± 723	*November 1991*	Marsh & Lawler (1992)
Torres Strait	27,881 ± 3216	*December 1995*	Marsh *et al.* (1997)
Great Barrier Reef region north of Cape Bedford	9,444 ± 1,381	*November 1995*	Marsh & Corkeron (1997)
Great Barrier Reef region south of Cape Bedford	1,642 ± 236	*November 1994*	Marsh *et al.* (1996)
Hervey Bay, Queensland	807 ± 151	*November 1994*	Marsh *et al.* (1996)
Moreton Bay, Queensland	850 total count	*December 1995*	Lanyon & Morrice (1997)

Table 2.4: Reports of dugong status outside of Australian and Torres Strait waters (adapted from Nishiwaki & Marsh, 1985).

Country	*Numbers*	*Reference*
Melanesia		
Papua New Guinea	186	Ligon & Hudson (1977) *1975 survey*
West New Britain	9	Hudson (1980) *1980 survey*
Manus island	41	Hudson (1981a) *1980 survey*
Micronesia		
Palau	26 (max. count.)	Marsh *et al.* (1995) *1991 survey*
Asia		
Palawan, Phillipines	15 (max. count)	Aragones (1994)
India		
north-western Sri Lanka	2 (max. count)	Hunnam (unpublished) *1981 survey*
West Indian Ocean		
Republic of Djibouti	30 (approx.)	Robineau & Rose (1982) *1980 survey*
northern Mozambique	27 (total count)	Hughes & Oxley-Oxland (1971) *1970 survey*
Kenya	8	Ligon (1976b) *1975 survey*
Arabian Gulf	7307 s.e. ±1302	Preen (1989b); Preen *et al.* (1989)

2.4.5 Life history

At maturity, dugongs are about 2.4 metres in length and weigh around 350 kg (Heinsohn, 1972; Marsh, 1980). The age of a dugong can be estimated by counting dentinal growth layers in its tusks/incisors (Marsh, 1980), which suggest a life span of about 70 years (Marsh, 1980; 1995). Sterile cycles in female dugongs appear to be common (Marsh *et al.*, 1984a; 1984c) and the time of reproduction varies regionally. Dugongs from the Townsville-Cairns area produce calves mainly between August and September (Marsh *et al.*, 1984c; Marsh, 1995), whereas at Daru (9°04'S, 143°21'E) most calves are born between July and February (Marsh, 1986; Marsh, 1995).

Reproduction in the dugong appears to be resource dependent (Marsh, 1995), and may also be affected by episodic mass mortalities and population density, as in some other marine mammals (Harwood & Rohani, 1996). For

example, a decreased proportion of calves was associated with the large-scale loss of seagrass from Hervey Bay, Australia (Preen & Marsh, 1995). Resource dependency may in part explain the recognised variation in the age of sexual maturity of dugongs which is around 10 to 17 years (see tables 1 & 2 in Marsh, 1995). The dugong gestation period is approximately 13 months, and the interbirth interval is around three to six years (see table 3 in Marsh, 1995). This variation could be temporal or spatial.

Dugongs usually have one calf at a time with a male to female ratio of 1:1 (Marsh, 1995). The high variability in age of sexual maturity and interbirth intervals, makes it difficult to estimate rates of population change and mean generation time. An approximate estimate of generation time can be obtained using the following equation (Begon *et al.*, 1990):

$$\text{generation time} = \frac{\sum x l_x m_x}{\sum l_x m_x}$$

Where x = age (years), l_x = proportion of female dugongs surviving to age x and m_x = the number of females produced per mature female at the age x. Using the information available on dugong reproduction (Marsh *et al.*, 1984a; 1984c) and age structure of dugong populations (Marsh, 1980), the dugong generation time is estimated to be 30 years. The estimate assumes: all females are breeding, their primary reproductive age is ten years, and the females remain active breeders for the remainder of their life. This estimate is approximate because of individual and regional variations in dugong life history (Marsh *et al.*, 1984a; 1984c). I have used the generation time of 30 years in my estimates of female migration between populations in section 8.4.

2.5 INTRASPECIFIC VARIATION

Dugong subspecies have been suggested. Historically, it was considered that dugongs from the Red Sea were morphologically distinct from those inhabiting shores and islands of the Indian Ocean, and those of northern Australia and New Guinea (Anderson & de Winton, 1902). However, these accounts and Gohar's (1957) proposal of the subspecies *D. d. tabernaculi*, are not supported by scientific evidence or reports of modified characters (Anderson & de Winton, 1902; Mohr, 1957; Spain & Marsh, 1981). Allen (1942) questioned the validity of a report by Dollman (1932), who compared

two dugong skulls from Mafia Island (east Africa) to an unknown number of Australian dugong skulls and concluded that the Australian dugongs were '...a larger and heavier animal'. No measurements were given by Dollman (1932), or any information about how the ages of the dugongs were verified.

Further reports of species subdivision in the dugong are few and anecdotal. Anderson (1995b) reported that Shark Bay (25°25'S, 113°35'E) dugongs were brown rather than slate in colour. Dugongs from the Philippines are apparently darker in colour and smaller than Australian dugongs (L. Aragones, personal communication). However, the significance of these anecdotal observations of regional colour and size variations is dubious given the lack of formal (colour) standards (H.D. Marsh, personal communication). Using 'questionnaire data', Nishiwaki *et al.* (1979; 1981) proposed a minimum of five distinct dugong 'populations' defined on the basis of alleged discontinuity of their distribution: 1) the east Australian and east Papua New Guinean group, 2) west Australia-Molucca-Philippine group, 3) Sumatra-Malaysia-Andaman group, 4) Indo-Sri Lanka group and 5) east African and Madagascar group. These 'populations' proposed by Nishiwaki *et al.* (1979; 1981) are not supported by any corresponding diagnostic characters.

The existence of species subdivision in the dugong has been more recently examined using scientific evidence by Spain & Marsh (1981) and Preen (1989b). Spain & Marsh (1981) demonstrated that the skulls of dugongs from the Wellesley Island group (16°41'S, 139°8'E) can be statistically separated from those from Townsville (19°15'S, 146°45'E). These two dugong populations from northern Australia are separated by approximately 1850 kilometres of coast. Preen (1989b) differentiated dugong skulls from the Arabian Gulf from dugong skulls from northern Australia (Spain & Marsh, 1981), and India (James, 1974). The Indian dugong skulls tended to have larger proportions relative to length than the Arabian Gulf skulls, which were heavier proportioned than the Australian skulls (Preen, 1989b). Variation in the skull morphometrics may be environmental, highlighting the utility of molecular tools to re-examine subdivision within the dugong. However, a consistent difference between localities in skull morphometrics is probably evidence against panmixis, as there is no indication that dugongs from widely dispersed areas aggregate to breed (H.D. Marsh, personal communication). Considering their vast range (section 2.4.2; figure 2.5), and

generally linear distribution along coastlines, intraspecific differentiation between dugong populations and the existence of species subdivision of the dugong would not be surprising.

2.6 WHY ARE DUGONGS AT RISK: THEIR EVOLUTION and MANAGEMENT

2.6.1 Anthropogenic impacts on sirenians

Sirenians are coastal species, and as a consequence are at particular risk from anthropogenic impacts. Since the Pleistocene (figure 2.3), at least 15 marine and coastal animal species have become extinct (Vermeij, 1993). Five of the 15 extinct marine and coastal species had large geographic ranges (Vermeij, 1993) challenging the widely accepted generalisation amongst conservation biologists and palaeontologists, that species with large ranges are not prone to extinction. All the coastal marine mammals of the Indo-West Pacific have large ranges (Klinowska, 1991). These include the finless porpoise (*Neophocaena phocaenoides*), Irrawaddy river dolphin (*Orcaella brevirostris*), Indo-Pacific humpback dolphin (*Sousa chinensis*), bottlenose dolphin (*Tursiops truncatus*), and the dugong. Genetic and morphological evidence suggests that these species comprise distinct non interbreeding populations, presumably as a result of their essentially linear habitats, which are fragmented by changes in sea level and current systems (UNEP, 1996). These species are listed by the IUCN (1996) as 'data deficient', with the exception of the Yangtze River population of the finless porpoise which is 'endangered', and the dugong which is listed as 'vulnerable to extinction'.

According to data compiled by the World Resources Institute (Bryant *et al.* 1995), much of the Indo-West Pacific region is threatened by coastal development. The future of the inshore marine mammal fauna is believed to be at risk (UNEP, 1996). Human pressure on the coastal zone includes high density settlement, fishing, deforestation and agriculture (Bryant *et al.* 1995; Zann, 1996). Factors contributing to the extinction risk of coastal marine mammals are their slow rate of reproduction (section 2.4.5), mass mortalities of local populations by natural disaster (section 2.6.6) and, perculiar to the dugong, a specialised diet (section 2.4.3). Extinction of the dugong would be a significant loss to marine biodiversity. In this thesis,

aspects of the population genetics of the dugong that might influence its survival have been investigated.

2.6.2 Vulnerability of Recent sirenians to extinction: A case study

Steller's sea cow grew to eight metres in length and weighed 10,000 kg (Scheffer, 1972). It was the closest Recent relative to the dugong (Domning, 1994). The rapid extinction of the Steller's sea cow is a classic example of the susceptibility of a slow breeding marine mammal to exploitation. It is one of the 15 marine species that became extinct since the Pleistocene, even though it had a large geographic range (Vermeij, 1993). Steller's sea cow is an example that supports the argument for preservation of the habitat, as well as the numbers, of a species vulnerable to extinction.

Naturalist Georg Steller was among the survivors of a Russian expedition shipwrecked in the Commander Islands off the coast of Siberia (North Pacific and Bering Sea) in 1741. There he found some 2,000 giant kelp-eating Steller's sea cows, the last of a species that had probably been eliminated by hunting throughout its vast former range (Stejneger, 1887; Domning, 1978). During the previous two million years, Steller's sea cows had lived along the Pacific rim from California to Japan (Domning, 1978). Steller's sea cow was ruthlessly hunted to extinction by 1768, a mere 27 years after its discovery (Stejneger, 1887). Steller's sea cow was the first marine mammal to become extinct in modern history.

Anderson (1995a) postulated a hypothetical scenario in which the evolution and distribution of Steller's sea cow was influenced by a kelp-urchin-otter relationship. It is likely that the Steller's sea cow competed with urchins for their common food source, shallow-water kelps. Sea otters preyed on urchins presumably keeping their numbers low. When the sea otters were hunted for their fur in the 18th Century, their numbers were reduced. Anderson (1995a) predicts that urchin numbers would have risen as a result, and grazed the shallow-water kelps. Otterless coastlines are relatively barren of boreal, shallow-water kelp communities. In addition, deep-water kelp species are known to invade areas where urchins have decimated shallow-water kelp species (as reviewed by Anderson (1995a). Deep-water kelps (e.g., *Agarum*) are toxic to herbivores. Steller's sea cow as a specialist feeder on shallow-water kelps, had an inshore habitat which made it easy to hunt. Because of its immense size, it could not dive and was not as elusive or agile

as the other sirenians. Along with the pressure from hunters, the extinction of the Steller's sea cow could have been promoted by a loss of their foraging habitat.

The history of the Steller's sea cow may be a forerunner to that of the dugong. Both species had their numbers reduced under the influence of anthropogenic impact. The Steller's sea cow was the dugong's closest related modern sirenian and shared similarities in biology and life history (e.g., large, slow breeding marine mammals), and a requirement for a high survivorship for species success. Both of these sirenians inhabit(ed) inshore coastal regions, had extensive ranges, and were specialised feeders.

2.6.3 Comparison of anthropogenic impacts to the Florida manatee and the dugong

In many areas, anthropogenic impacts on sirenians include: hunting pressures, motorboat strikes, netting, and habitat damage due to pollution and loss of habitat area. These factors are likely to cause dugong mortalities, and influence dugong movements and distribution. The most likely effects are habitat fragmentation, a reduction in dugong numbers, and a possible loss of genetic diversity, which may hamper species recovery by reducing evolutionary potential (discussed in Chapter 12).

The Florida manatee, one of the dugong's closest extant relatives, has been studied more intensively than the dugong, and shares a number of similarities in biology and life history. Furthermore, the Florida manatee is subjected to some of the same anthropogenic impacts as the dugong and has similar conservation concerns. For these reasons, the Florida manatee provides an ideal comparative case. On the other hand, the Florida manatee may not be ideal as a comparative species for the dugong, because it has a different (fresh water dependent) less continuous habitat. The manatee's habitat extends from the sea to inland waterways, right into the backyards of Florida residents. The callenges the manatee has faced in their urban adaptation include exposure to a wide range of anthropogenic impacts, including entanglement in fishing gear, crushing and/or drowning in canal locks or flood control structures.

2.6.4 Motorboat traffic

The greatest cause of mortality to Florida manatees is motorboat collisions (Wright *et al.*, 1995). Despite speed regulations and pleas from conservationists, more than 150 manatees die annually due to watercraft collisions. Manatees have the physical ability to evade boats yet most individuals bear the scars of repeated encounters (Beck *et al.*, 1982; Reid *et al.*, 1991; Gerstein, 1994).

Anecdotal evidence indicates that noise pollution (from engines) and boat traffic also affect dugong movements (Preen, 1993; Peterken, 1994). However, dugongs that are not routinely hunted or harassed, will display curiosity towards boats, by approaching and circling vessels that are drifting or anchored (Anderson, 1982; personal observation). Scars on dugongs attributable to boat propellers, or records of boat strike as the cause of death, are extremely rare (Anderson, 1982; Anderson, 1995b; H.D. Marsh, personal communication). The openness of dugong habitat, the lower levels of boat traffic along the north Australian coastline compared to Florida, and the behaviour of dugongs, may make them less susceptible to boat strike than manatees (Anderson, 1982). However, as most seagrass beds occur in shallow bays, the potential impact on dugong movements and numbers of increased motorboat traffic, associated with the increasing coastal development, should be seriously considered.

2.6.5 Shark and gill nets

Another major cause of incidental dugong deaths from anthropogenic activities is drowning in shark or gill nets as a result of accidental entanglement (figure 2.6; Bertram, 1981; Marsh, 1988). Both commercial mesh netters and dugongs utilise the intertidal areas on high tide, which increases the probability of accidental netting of the dugongs (Marsh & Corkeron, 1997). Anecdotal evidence of the bycatch of dugongs in commercial mesh nets suggests that it is not sustainable in the Great Barrier Reef region. For example, of 14 dugong carcasses reported to management agencies between April and September 1996, at least eight were probable net kills (J. Slater (personal communication) *in* Marsh & Corkeron (1997)). Few dugongs are drowned in commercial prawn trawls (Marsh *et al.*, 1996).

A summary of non-target species caught by shark nets along the Queensland coast between 1962 and 1991, includes 837 dugongs (Anonymous, 1992).

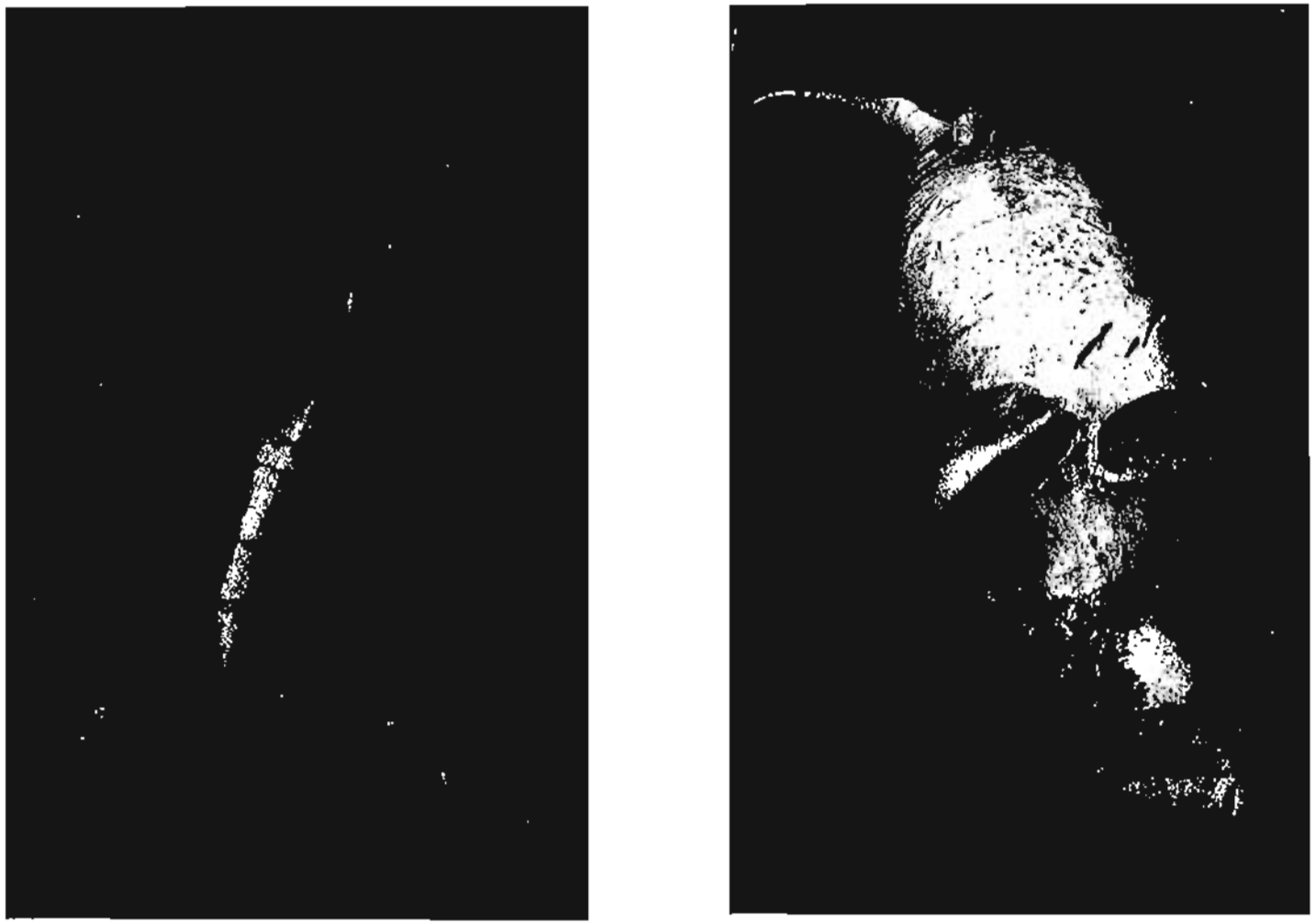


Figure 2.6: A male dugong drowned by a gill net in Townsville (sample 329, see Appendix C for tissue bank records). Note the rings of bruises across the abdomen caused by the gill net.

Only 2.4% of the dugongs were recorded as being released alive. The captures of dugongs in shark nets set off Townsville increased from 12.7 dugongs annually before cyclone *Althea* in 1971, to 41 after the cyclone. Heinsohn & Spain (1974) attributed the increase in capture in shark nets, to the dugongs moving in search of food. Since the shark netting program was reorganised in 1992 by replacing many nets with baited hooks, five dugongs have been netted in the region of the Great Barrier Reef (Marsh *et al.*, 1996). However, the replacement of nets with drumlines does not completely eliminate the bycatch of megafauna. Loggerhead turtles (*Caretta caretta*) have been known to be caught on drumlines set as part of the Shark Protection Program in southern Queensland (Marsh *et al.*, 1996).

2.6.6 Mass mortalities, habitat damage and loss

Mass mortalities from natural impacts are well recognised for the Florida manatee. About 200 Florida manatees died in early 1996 (Kleiner, 1996) due to a toxic Red Tide algal bloom. As the manatees of Florida were already at risk because of watercraft collision and loss of habitat from coastal development (prior to the bloom manatee numbers were estimated to be around 2,600), this additional stress was of real concern, despite the widening body of opinion that Florida manatee numbers are generally increasing (Ackerman, 1995).

The clearing of forests, mining and sewage effluent, pesticide, fertiliser and sediment runoff from agricultural land, and intensification of fishing and boating activity, all contribute to pollution and the destruction of seagrass beds. In addition to hunting of the dugong as a resource, these factors are likely contributors to localised extinctions of the dugong in parts of their former range.

Intense storms/cyclones are known to affect the mortality of dugongs by extensive damage to sea grass beds on which dugongs feed (Heinsohn & Spain, 1974). An example of devastation of dugong habitat by extreme weather events is the unusual combination of two major floods and a cyclone in Hervey Bay, Australia in early 1992 (Preen & Marsh, 1995). The increased human settlement and agriculture along the rivers and creeks feeding the Bay are likely to have contributed to this massive destruction of seagrass. Preen & Marsh (1995) estimate that approximately 1,000 dugongs left the area. Dugong carcasses were found up to eight months after the

event, with 99 dugong carcasses being recorded. This is likely to be an underestimate of the actual number of dugongs that died. Most of the dugongs died of starvation as suggested by the emaciated carcasses. Preen & Marsh (1995) estimate that in the absence of immigration, it will take more than 25 years for dugong numbers in the bay to recover. This demonstrates the requirement to consider the potential impacts of habitat loss or modification, as well as the influence of traditional hunting and incidental mortality in shark and gill nets, when managing for the conservation of dugong populations (Preen & Marsh, 1995).

2.6.7 Indigenous hunting and commercial exploitation in Australia

Australian Aborigines and Torres Strait Islanders continue to hunt dugongs for subsistence in Australian waters. To them the dugong is more than an important food source. It is central to their culture, economy and religion (Chase, 1981). Methods of dugong hunting and the significance of the dugong to the culture of Torres Strait Islanders have been extensively documented by Johannes (1981) and Johannes & MacFarlane (1991).

Dugongs were hunted in Australia from the mid-1800s to the 1940's, as their oil was believed to cure symptoms ranging from joint pains and tuberculosis to hair loss. Frustrated by a lack of reliable supplies, the dugong 'fishery' in Moreton and Hervey Bays had limited success and did not eventuate to more than a cottage industry (Peterken, 1994). However, it is unknown whether these populations have recovered to their 'prefishery' size.

2.6.8 Australian protective legislation

The dugong is listed in the IUCN Red Data Book of Threatened Species as 'vulnerable to extinction' (IUCN, 1996). Australia has international obligations for dugong conservation, as the Australian government has ratified the following international conventions: *The Convention on International Trade in Endangered Species of Wild Fauna and Flora 1973* (CITES); *The Convention on the Conservation of Migratory Species of Wild Animals 1979* (the Bonn convention); *The Torres Strait Treaty 1985*; and *The Convention for the Protection of the World Cultural and Natural Heritage 1972* (World Heritage Convention). In addition, Australia has a number of Commonwealth Acts which address these international obligations as detailed in Marsh *et al.* (submitted). In particular, the import and export of

any dugong specimen or product (other than faeces) from any international or national dugong population is regulated under Schedule II of the *1982 Australian Wildlife Protection Act* (obligation to CITES).

Marsh *et al.* (submitted) presents details of the Australian Commonwealth legislation, and the independent legislation of Western Australia and Queensland, and the Northern Territory. The Acts include: establishment and management of marine protected areas, or marine conservation reserves; prohibition of hunting (capture and slaughter) of dugongs by non-indigenous Australians, unless under a scientific research permit; and traditional indigenous use of the dugong under certain conditions. The legislative background is well developed in Australia. However, it is not well policed (Roberts *et al.*, 1996).

Both the Great Barrier Reef Marine Park Authority (GBRMPA) and the Queensland Department of Environment, have consulted with Aboriginal and Torres Strait Islander groups for over a decade regarding dugong hunting. In the southern Great Barrier Reef, dugong numbers have fallen by 50% between 1986/87 to 1994 (Marsh *et al.*, 1996). As a result of this concern, the *Darumbal-Noolar Murree Aboriginal Corporation for Land and Culture of Rockhampton* signed a formal agreement with GBRMPA, to refrain from hunting the dugong within the Shoalwater Bay Military Training Area (Marsh & Corkeron, 1997). Other indigenous councils (south of Cooktown) have voluntarily agreed not to harvest the dugong, as they have become aware of a regional decline in dugong numbers.

Legislation also exists to protect dugongs in countries other than Australia, however it is not necessarily effective, as governments often lack funds or resources to protect their wildlife.

2.6.9 Conclusion: Requirement for conservation management

Native to the coastline of more than 40 tropical and subtropical countries, the dugong is considered rare throughout its extensive range, and is in some areas, critically endangered. Australia and the Torres Strait apparently support the largest dugong populations in the world. However, even in some parts of Australia, the dugong's existence is threatened. Anecdotal evidence suggests that along the east coast of Australia, dugongs have been declining since the 1960s or earlier. In the last eight years, this decline has

been shown to approach 50% in a large section of the Great Barrier Reef (Marsh *et al.*, 1996).

Even in the absence of natural catastrophes and further anthropogenic impacts, recovery of the dugong population size is expected to be gradual because of the dugong's low reproduction rate. Steller's sea cow provides an example of the vulnerability of large slow breeding marine mammals to extinction, even if their ranges are large. The dugong has many similarities to the Steller's sea cow, including taxonomic closeness, coastal habitat and susceptibility to anthropogenic impact. The greatest threats to the existence of the dugong are traditional hunting, drowning from net entanglement, and the destruction of dugong habitat.

In Australia, the majority of human settlement is coastal with around 80% living in the coastal zone, 25% within three kilometres of the sea (Zann, 1996). The ocean is used for purposeful and incidental dumping of a number of human waste products. Sewage, sediment, fertiliser and chemical runoff, feed into and concentrate in bays harbouring dugong habitat. In addition, tidal surges and storms dump flotsam and jetsam (plastics, fishing line, ropes) into coastal areas. Fishing and recreational activity introduce noise pollution, nets, and marine vessel traffic. These are some examples of anthropogenic impacts to dugong habitat, all of which would undoubtedly affect dugong movements and distribution, and even their survival. Preserving a healthy coastal environment is an essential prerequisite of dugong survival in the future.

As a developed nation, Australia should have the resources required to protect our wildlife. We have to take the responsibility and set an example to other countries to conserve the dugong. Otherwise, as E.J. Banfield *in* LeSouef & Burrell (1926) predicted, 'Dugongs are certain of extinction, as all their haunts are ruthlessly ravaged'.

2.7 PREDICTIONS: EXPECTED GENETIC PATTERNS

The marine environment has high connectivity, however there are at least three habitats that are easily recognised: pelagic, coastal and land/coastal. Whales are often characterised as having a pelagic habitat with a nearly or completely cosmopolitan distribution. They have enormous migration and

dispersal ability. Sirenians and some cetaceans (section 2.6.1) occupy coastal habitats that include inland waterways. The habitat of pinnipeds includes both shoreline breeding colonies and inshore waters. Barriers to marine mammal movement are not as obvious as they are for land mammals. Distinct genetic units can arise in the situation of reproductive isolation, thus it would be expected that mobile pelagic species would be less likely to display distinct intraspecific genetic units. Genetic diversity is predicted to be low for small populations that have undergone bottlenecks. Conversely, high genetic diversity is predicted for large populations that maintain a relatively constant size. Of course, genetic diversity is not driven purely by population size; selection pressures from the environment as well as the biology of the species also play active roles.

Food availability and distribution are expected to govern dugong movements and their range, hence possibly also their genetic structuring. With a linear, and nearly continuous range around the tropical and subtropical Australian coast, it is expected that if any intraspecific genetic differentiation exists for the dugong, it will be greatest between populations living at opposite ends of their range. However, considering that a dugong is capable of swimming hundreds of kilometres in a few days (Marsh & Rathbun, 1990; A.R. Preen, personal communication), the possibility of genetic homogeneity among populations should not be excluded. The level of intraspecific genetic structuring for the dugong is expected to lie somewhere between the extremes of the distinct genetic groups typical of many seal colonies (e.g., Hoelzel *et al.*, 1993a; Cronin *et al.*, 1994; Stanley *et al.*, 1996), and the large homogenous units characteristic of some pelagic cetaceans (e.g., Dizon *et al.*, 1991; Hoelzel & Dover, 1991b). Where they fall in this range will largely be influenced by dugong behaviour and social structure.

CHAPTER THREE

SAMPLE COLLECTION

A major part of this study was collecting samples and extracting DNA from tissue of varying quality. The dugong tissue bank was developed from three sources: 1) samples collected by marine rangers and community members from dugongs hunted by Aborigines and Torres Strait Islanders, 2) samples collected via necropsies of dugong carcasses that washed up on beaches, and 3) biopsy sampling and faecal collection from free ranging dugongs. This chapter details each of these collection approaches. The people who helped with sample collection are listed in Appendix A.

3.1 SELECTION OF TISSUE FOR DNA EXTRACTION

Any tissue that contains DNA can be amplified using the PCR (section 4.3). The only dugong tissue from which I have not been able to isolate DNA, is the hypodermis (fatty white fibrous layer) of skin. I did not invest much effort in trying to amplify DNA from formalin-fixed tissues, as I had access to samples preserved in ways that did not cross-link the DNA. The best DNA source was fresh tissue (i.e., not decomposed), or tissue that was preserved in a medium (e.g., ethyl alcohol, DMSO) that did not cross-link the DNA.

Faeces and bone were not routinely collected. However when samples were difficult to obtain from a particular geographical area, faeces or bones present on local beaches (e.g., Aboriginal middens) were collected. DNA has been successfully extracted from dugong bone, and extracted, amplified and sequenced from dugong faeces (Tikel *et al.*, 1996, Appendix B). Only one sample from dugong faeces has contributed to the data set for this study (figure 3.1; Appendix C) representing Borroloola (figure 3.2). Most samples were from muscle or skin (Appendix C).

3.2 PRESERVATION OF SAMPLES

Because formalin cross-links DNA, any tissue in preservative containing formalin is difficult to amplify using the PCR (table 3.1). Although it is

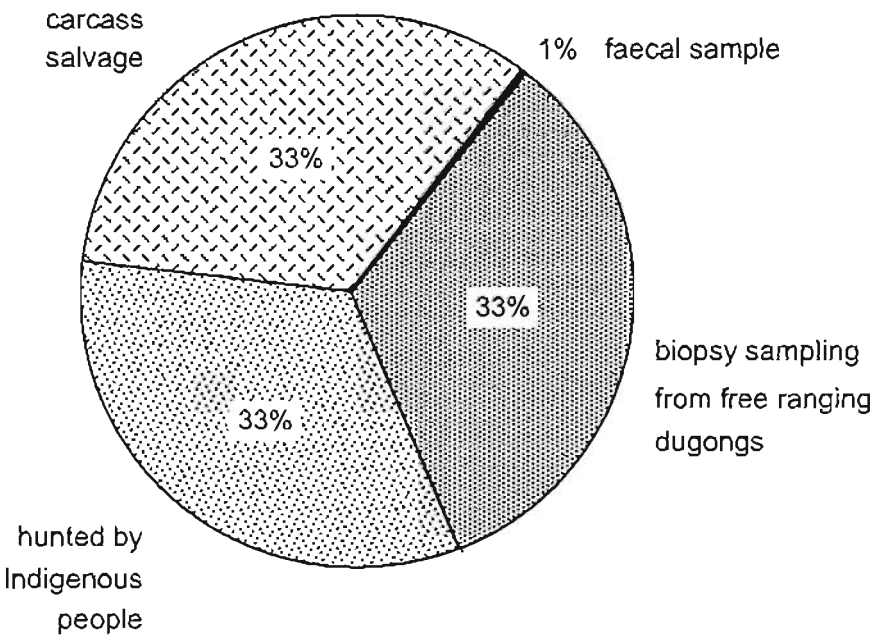


Figure 3.1: The proportions of samples collected via the various methods. Only samples that were sequenced and used in the analysis are included (N=103). A complete listing of all samples is indicated in Appendix C of which the method of collection was not always known.

possible to extract and amplify DNA from formalin-fixed, paraffin-embedded tissues (section 2.2.3), I do not recommend these tissues as a source of DNA.

Table 3.1: **Inhibitor effects on *Taq* polymerase I activity** (Erlich, 1989).

Inhibitor	*Concentration*	*Activity**
Ethanol	≤ 3%	100%
	10%	110%
Urea	≤0.5M	100%
	1.0M	118%
	1.5M	107%
	2.0M	82%
DMSO	≤1%	100%
	10%	53%
	20%	11%
DMF	5%	100%
	10%	82%
	20%	17%
Formamide	≤10%	100%
	15%	86%
	20%	39%
SDS	0.001%	105%
	0.01%	10%
	0.1%	<0.1%

*dNTP incorporation activity at 70°C with Salmon Sperm DNA/10 minutes.

Both 70% ethyl alcohol and 20% DMSO in saturated NaCl, are excellent preservatives. Ethyl alcohol preserves tissue by 'dehydration', whereas DMSO 'punches' holes in the cell membrane allowing NaCl to preserve the tissue. DMSO is considered superior to alcohol as a preservative for the following reasons:

1) Unlike ethyl alcohol, DMSO is not registered as sufficiently toxic or flammable (flash point of 95°C) to require a United Nations hazardous chemical code. Thus, there are no restrictions to its postage or transport on aircraft.
2) DMSO evaporates much less rapidly than ethyl alcohol.

I often received vials of tissue that were dried, despite being originally immersed in ethyl alcohol. These samples are usually still well preserved, particularly if the original ratio of the tissue to ethyl alcohol volume was low.

Other methods of preservation include drying or freezing the tissue. Drying tissue by enclosing sacks of a dehydrating agent within the vial is an increasingly popular means of preservation of tissue, particularly for transport. As only small thin strips of tissue preserve well with this approach, I have not chosen to preserve dugong tissue this way. Freezing tissue although ideal, is awkward in the field. Keeping the sample frozen in transit (e.g., via dry ice) is costly, cumbersome, and impossible for collection of samples from remote areas.

Although I recommend collection of dugong tissue in DMSO and continue to use and recommend this preservative medium, the dugong tissue bank developed as part of this study includes material preserved in all of the ways described above (Appendix C). All samples, regardless of the preservative, were stored at 4°C (if contained in a preservative medium) or -20°C (if not in a preservative medium). The level of interference of various preservatives in the PCR is shown in table 3.1.

3.3 PERMITS

Biopsy samples were collected under Fish or Marine Products Permits # 4418, # 4784, and #5112 issued by the Queensland Department of Primary Industries. Dugong samples from the Cairns section and Great Barrier Reef Marine Park, were collected under Permit # G92/492 issued by the Great Barrier Reef Marine Parks Authority and the Queensland National Parks and Wildlife Service.

3.4 SAMPLE COLLECTION

Establishing a sample collection network and dugong tissue bank (Appendices A & C respectively), was a gradual process facilitated by the contacts of H.D. Marsh, other dugong researchers and marine rangers (Appendix A) with access to free ranging and/or captive dugongs (e.g., Toba Aquarium, Japan). Dugong samples sequenced in this study, were collected

from the geographic locations specified in figure 3.2. Efforts have been made to maintain the sample collection network and to develop the tissue bank, for future genetic studies.

The sample collectors were requested to obtain samples using sterile blades and individual containers. Using disposable items when handling dugong tissue limited the risk of cross contamination, for example, from humans (via handling), or from bacteria and yeasts (from the environment). The use of species-specific primers to amplify the DNA (via the PCR, section 4.3) and the use of large volumes of the DNA of interest, reduced the risk of amplifying the non specific product.

Collectors were supplied with a sample kit (Appendix D) and instructed to select a strip of the grey epidermal layer of skin, or one cubic centimetre of muscle. Vials containing the preservative 20% DMSO in saturated NaCl (Amos & Hoelzel, 1991), were supplied in the kit. DNA was extracted from the tissue according to the protocol outlined in section 4.1.

3.5 MANATEE SAMPLES

Several specimens from Florida manatees were obtained from M.C. Milinkovitch & S. Wright (details in Appendix C). The mitochondrial DNA (mtDNA) from one of these specimens was sequenced and included in the analysis of Chapters 6 and 7 for comparison as an outgroup.

3.6 SAMPLES FROM DUGONGS HUNTED BY INDIGENOUS PEOPLE

Of the samples collected from Torres Strait (n=60), Starcke River (n=9) and Western Australia (n=20) (figure 3.2), 83% were obtained from dugongs hunted by Indigenous Australians (figure 3.1; Appendix C). Modern methods of hunting involve the use of a wooden canoe or aluminium dinghy with an outboard motor. The dugong is struck with a hand held harpoon (*wap*) and drowned (Patterson, 1939; Marsh *et al.*, 1981; Johannes & MacFarlane, 1991). The collector of samples (usually a community ranger) then cut a section of skin and/or muscle, placed it in a polycarbonate vial containing preservative and sometimes recorded on a data sheet various

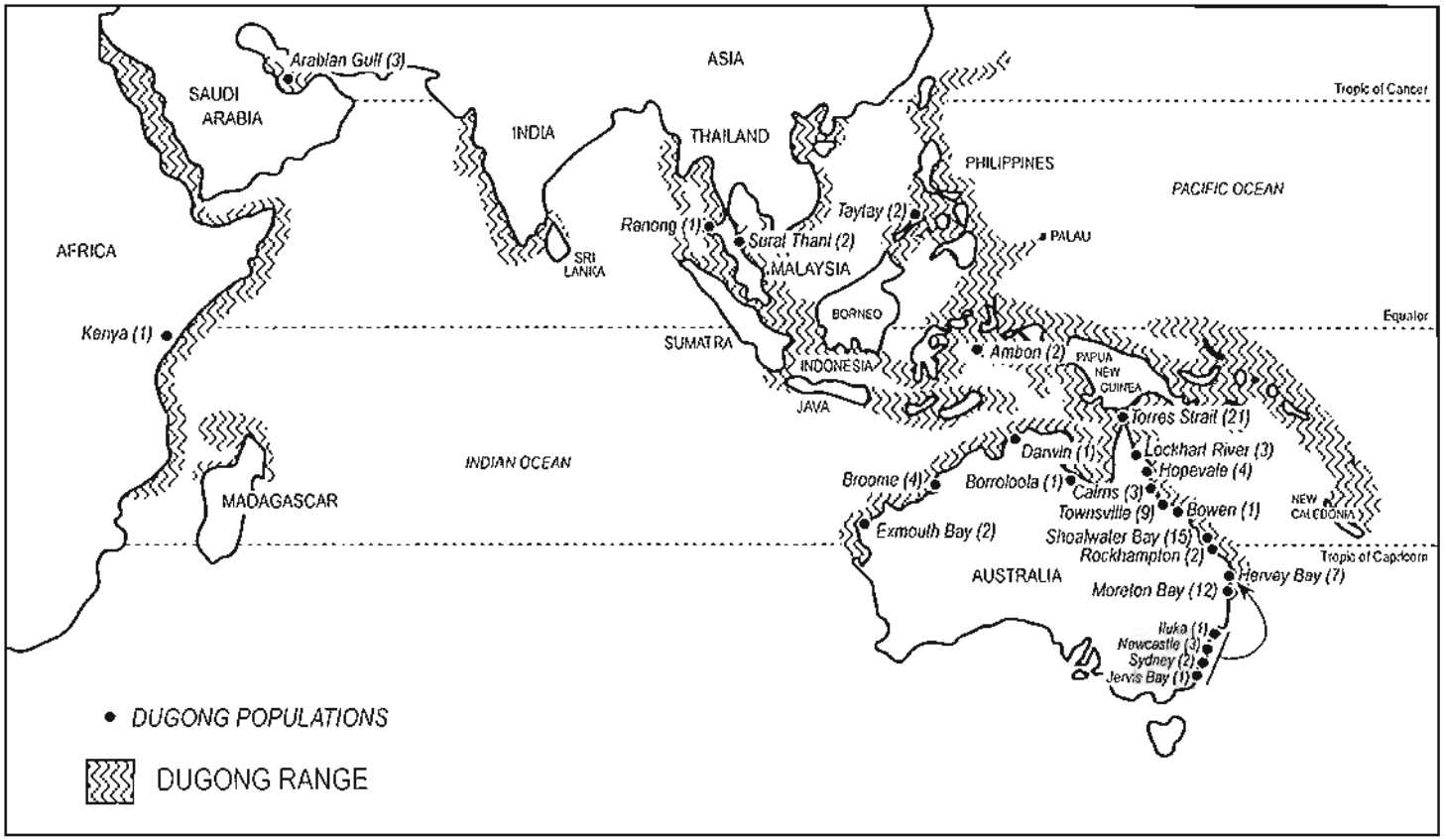


Figure 3.2: Dugong range (Marsh & Lefebvre, 1994) and sample locations (populations). Sample size collected from each location is indicated within brackets. Samples outside of the dugong's range along the New South Wales coastline (Australia) were presumed to be originally from the Hervey Bay dugong population as indicated by the arrow, because of their genetic similarity and the time of sample collection (see section 7.3.2.2 for justification of this decision).

details about the dugong. If data were recorded, it was usually a measure of body length and gender (Appendix C).

3.7 CARCASS SALVAGE

When a dead dugong was found washed ashore onto a beach along the Australian coast, a full or partial necropsy was carried out and samples were collected. These dugongs had usually died from natural causes or drowned after being trapped in gill or shark nets set for bather protection (section 2.6.5, figure 2.6). Most of the samples collected along the east coast of Australia south of Cape York, were obtained via carcass salvage (Appendix C), particularly near coastal areas with intense human settlement.

The amount of information that can be obtained from a carcass is inversely proportional to the time between death and necropsy. If the carcass is fresh, a necropsy involves taking length measurements (Appendix D), skin and muscle samples for this DNA study, heart, liver and other organs for parasites, stomach and intestine samples for a diet study, the reproductive system if it is a female (uterus and ovaries plus foetus if present), and the skull for morphometrics and age determination (using the incisors). If the carcass is decomposed (bloated or exploded), the necropsy involves taking length measurements, the collection of skin, muscle (if not too decomposed), and the skull. The skin is usually the best preserved tissue, perhaps because of its exposure to the sun and salt, which dries it out. Bone (marrow) is also well preserved. However, bone was collected only if the skeleton was the only tissue available. Only two of the 254 samples were bone (Appendix C).

3.8 BIOPSY SAMPLING OF FREE RANGING DUGONGS

3.8.1 Introduction

Sampling from the free ranging population was essential to obtain adequate representatives from particular areas of the dugong's range (particularly the east Australian coast). DNA sources available from a free ranging population of most mammals includes: hair, blood, faeces and skin biopsies.

Faecal collection is a non invasive procedure that is easy and economical. DNA extraction and sequencing from a faecal sample in the dugong is a recent development (Tikel *et al.*, 1996, Appendix B). At present, this approach does not provide a superior sample, because greater time investment and expertise in the laboratory is required to prepare the sample for sequencing. In a population level study, the possibility of resampling the same individual should be treated statistically as the same as taking skin biopsies from a free ranging dugong population. If collection of faeces to extract DNA is used to study familial patterns, observation (in conjunction with a tagging and/or photo-identification) is required. Because faecal collection is the least invasive method of obtaining DNA from a species of interest, it is likely to become more popular and refined in the future.

To obtain hair, blood and skin biopsies, a free ranging dugong has to be approached, caught and handled, a procedure that requires considerable expertise and also risks death of the dugong because of stress (Heinsohn *et al.*, 1976; Marsh & Anderson, 1983; Marsh, 1986). The alternative is to obtain a skin biopsy (e.g., by firing bolts with attached biopsy heads from a crossbow) from a vessel, a technique routinely used on cetaceans (Brown *et al.*, 1991; Palsbøll *et al.*, 1991; Weinrich *et al.*, 1991). As detailed below, this approach was trialed with some success in the dugong, but was not as successful as a biopsy-device that was specifically designed to sample dugongs (section 3.8.4.3).

3.8.2 Collection sites and location of dugongs

Sampling within Moreton Bay (figure 3.2) was performed during the Australian winter to maximise the chance of favourable weather conditions. Only dugongs from within the bay were targeted, as the dugongs residing outside of the Bay (typically on the ocean side of Moreton Island taking advantage of the warm currents) were difficult to find in the deeper water. It took a few days to elucidate the pattern of dugong movements within the bay and to find their apparent favoured feeding grounds (seagrass beds) using nautical charts and guidance from A.R. Preen and C.J. Limpus.

3.8.3 Vessels

Two vessels were used for the remote collection of samples, a 5.79 m motorboat and a 4.65 m rigid hulled 'Avon', with 150 and 50 horsepower outboard engines, respectively.

3.8.4 Trials with various biopsy equipment

3.8.4.1 Crossbow & Bolts

The initial approach used to target dugongs, was by firing bolts with attached biopsy heads (Brown *et al.*, 1994) from a 'Barnett' crossbow (150 lbs draw weight; figure 3.3).

When a herd of dugongs was found, the vessel was allowed to drift amongst the group to ensure they would not be disturbed by engine noise. Sampling opportunities were rare with this approach. Dugongs often surfaced exposing just their nostrils to breathe. As a dugong deep-dived, the sequence of exposure was head, back, then tail. A biopsy attempt was only made on a diving dugong, as the back provided the safest and longest exposed target. To avoid having to swim to retrieve bolts (if the vessel's engine was started, the noise dispersed the dugong group), a fishing reel with line was linked from the bolt to the crossbow.

The favoured range for targeting a dugong with the crossbow was between 3 and 12 m. Firing the crossbow at a range less than 3 m was not attempted in case the force of the bolt was too powerful, risking penetration beyond the stopper. Beyond 12 m the flight passage (height) of the bolts dropped rapidly.

Sampling using a crossbow and bolts with attached heads for taking biopsies was only partially successful. Five samples were collected using the biopsy heads during a total of at least 11 attempts over at least five expeditions. Biopsy heads were cleaned by scrubbing and rinsing with detergent and/or alcohol between biopsies. Two samples were useless as they consisted of hypodermis only and failed to yield DNA (see section 3.8.6 for discussion of dugong dermal structure). If the biopsy head glanced off the dugong's back, sufficient skin was usually obtained. However, when the biopsy head hit perpendicular to the dugong's back, the bolt tended to bounce without taking a plug. Occasionally the internal barbs of the heads would retain a

Figure 3.3: Obtaining biopsies from free ranging dugongs. A biopsy head (Brown *et al.*, 1994) was screwed into the end of a bolt with polyurethane foam (coloured orange) to ensure that it would float. The bolt was then loaded onto a 'Barnett' crossbow with a 150 lbs draw weight. The favoured range for targeting a dugong with the crossbow was between three and 12 metres.

scrap of tissue. Additional biopsy heads were produced with slight variations in design (Brown *et al.*, 1994) and tested for their capacity to puncture and take a plug of tissue, both in the field and upon a dugong carcass. Usually the bolts bounced, leaving a circle of penetration without taking a plug.

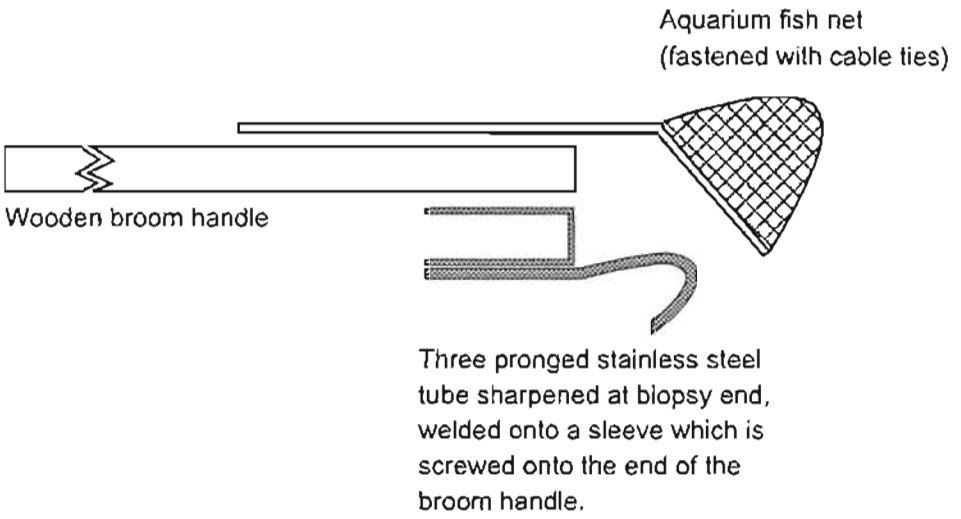
3.8.4.2 Hand spear

Waiting for sampling opportunities with the crossbow was time consuming, therefore an alternative approach for taking biopsies from dugongs was developed. Using a modified version of the technique used by Aborigines and Torres Strait Islanders to hunt dugongs (Marsh *et al.*, 1981; Johannes & MacFarlane, 1991), the biopsy heads were detached from the bolts and placed onto the end of a hand spear. The armed person was positioned at the bow of the vessel (the 4.65 m rigid hulled 'Avon' with a 50 horsepower outboard) and with the help of a driver and spotter, a dugong was herded into shallow water and biopsied when it surfaced. Dugongs surfaced more often than usual when chased by a vessel providing more sampling opportunities, but care was taken never to chase the dugong to exhaustion. Operating the biopsy heads with a hand spear was a much faster and a more productive approach to obtaining biopsies than with the crossbow.

3.8.4.3 Biopsy device

As the biopsy heads attached to the end of a hand spear were not usually successful in obtaining a full plug of tissue, the biopsy system was further modified to a device composed of three 'fingernail' prongs which was scraped over the dugong's back (figure 3.4). This device has a net positioned to catch any pieces of skin which came away from under the prongs (figure 3.4). The fingernail prongs take superficial strips of the pigmented (grey) epidermal tissue and do not become embedded. The technique should be no more invasive than superficial wounds caused by the tusks from vigorous interactions between dugongs (Preen, 1989a).

This method of collection proved to be the most time and cost effective method of collecting biopsies from free ranging dugongs. Expenses were further reduced by combining dugong sampling with turtle sampling expeditions by C.J. Limpus, who employs operators with experience in handling large and highly mobile marine animals.



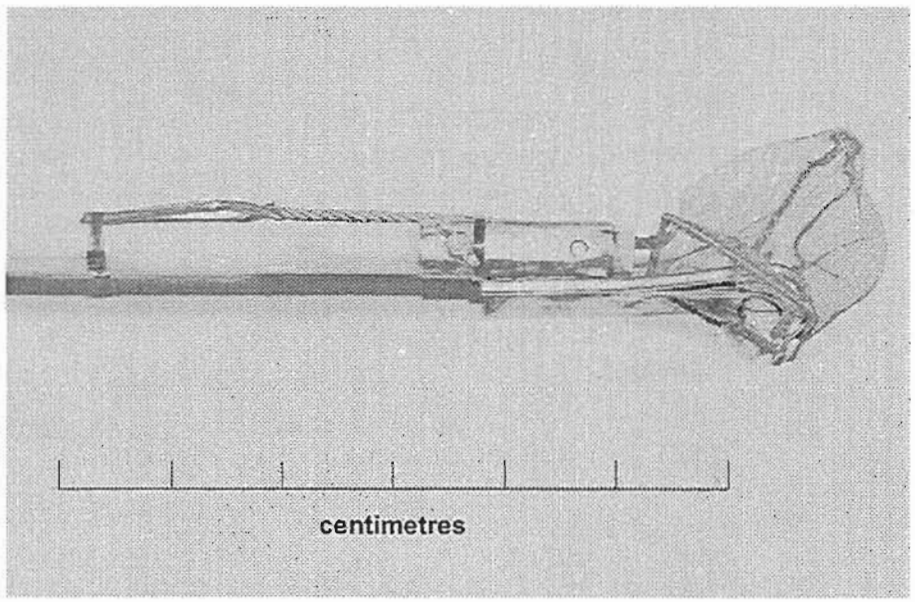


Figure 3.4: Side view of the biopsy device.

3.8.5 Response of dugongs to biopsy sampling

When a dugong was hit with a bolt fired by the crossbow, it responded with quick vigorous flicks with the tail followed by a dive. A similar response was observed when a bolt was fired and the dugong was missed. The dugong herd did not disperse during a sampling incident, even when I snorkelled to retrieve the bolts.

3.8.6 Discussion and Conclusions

Bryden *et al.* (unpublished) observed that the skin surface of the dugong has '...few specialised sensory endings...and it is uncertain what sensory function this [lack of sensory endings] serves, if any'. I do not suggest that a dugong does not feel the impact of a biopsy head. However I do propose that the lack of sensory endings on the superficial layers of the skin, indicates that the impact of biopsy heads may not be as traumatic to the dugong as may be anticipated. Certainly it is no more invasive than tusk injuries caused by battles between dugongs (Preen, 1989a).

The system developed specifically for the dugong, a three pronged device which is scraped over the dugong's back (figure 3.4) and took an epidermal biopsy, gave satisfactory results. This method of collection has reduced the time and cost of sample collection from free ranging dugongs whilst being minimally intrusive.

The lack of success of biopsy methods popular for sampling cetaceans, when applied to the dugong, is indicative of a difference in their dermal structure. The biopsy heads used in this study were successfully used to biopsy humpback whales (*Megaptera novaeangliae*) (Brown *et al.*, 1994) and consistently took a full plug of tissue. The dugong has a one millimetre thick grey epidermal layer superficial to a 10 mm (minimum thickness) white 'blubber' dermis (figure 3.5; Bryden *et al.*, unpublished). The dermis is composed of dense collagen which makes the taking of a biopsy difficult. Bryden *et al.* (unpublished) stated '...connective tissue components of the skin seem to be arranged to permit maximum flexibility of the skin superficially (many elastic and reticular fibres in the papillary layer), possibly allowing slight alterations in shape of the surface layers during swimming, while retaining considerable rigidity in the deeper layers...'. Whale skin has a 'papery' upper layer of dead cells flaking off over the live epidermal layer, which overlies the blubber. When hit, cetacean skin is 'hard'; the darts

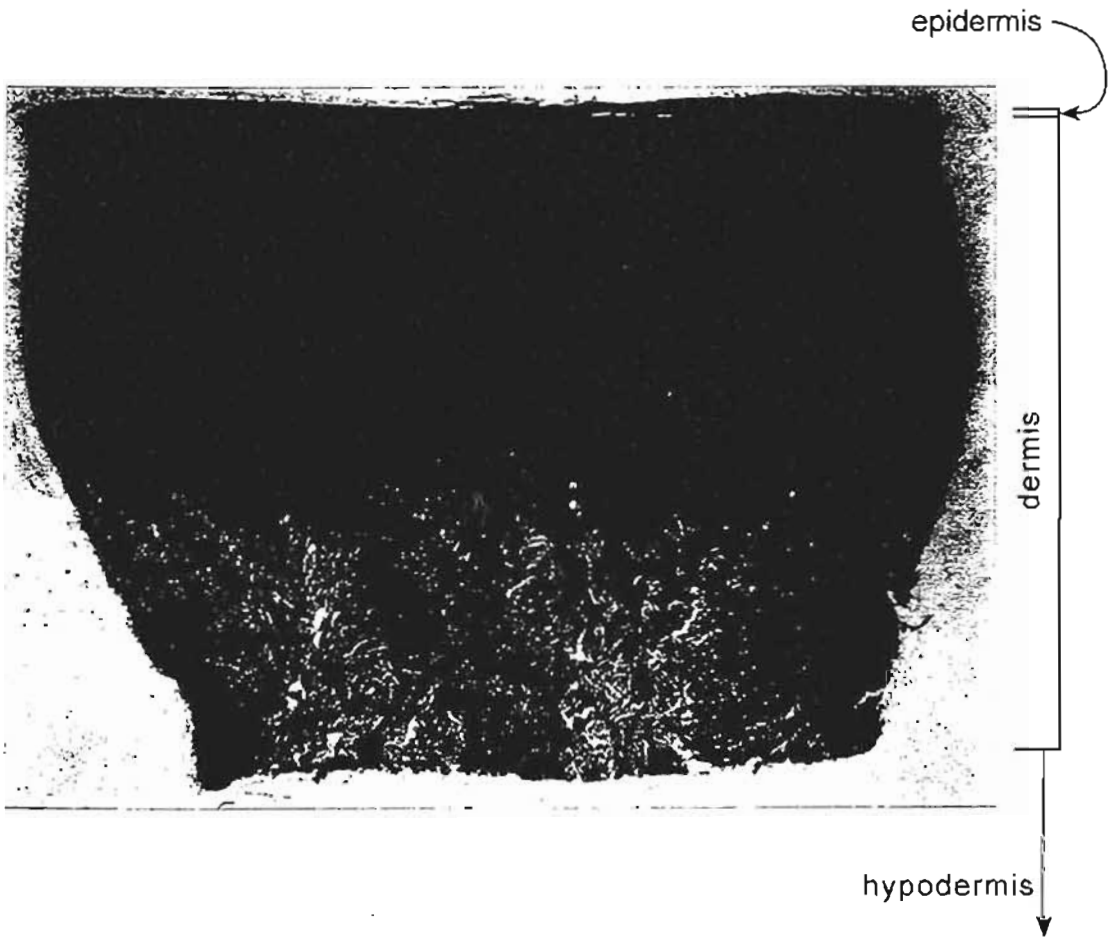


Figure 3.5: **Dermal structure of the dugong.** Section of dugong integument (skin) stained in *Mayer's haematoxylin* and *Young's eosin-erythrocin* solution. This stain highlights nuclear and pigmented tissue. The most external layer of skin (epidermis) is grey in colour and is about 1 mm thick. The epidermis consists of three layers as in many other marine mammals: stratum corneum, stratum spinosum, and stratum basale (Bryden et al., unpublished; 1978). The epidermis is underlaid by the dermis which is more than 10 mm thick, white in colour (when not stained), and composed of dense collagen. The dermal layer is not invaded extensively by fat cells, and overlies the hypodermis. The papillary layer of the dermis is stained here in dark pink (dense with nuclei), and overlies the reticular layer of the dermis which is stained lighter pink and dense with collagen.

bounce, taking a full plug of tissue (P. Hale, personal communication). In contrast, when a dart penetrates the dermis of a dugong, it tends to 'hang on'. Torres Strait Islander dugong hunters reported that a dugong's skin is thick, and that a *wap* (hand held harpoon) seldom penetrates vital organs (Johannes & MacFarlane, 1991). Furthermore, Torres Strait Islander hunters say dugong skin is 'slack' when they feed, but tightens when they are speared, and when the spear head does penetrate, it must be cut out (Johannes & MacFarlane, 1991).

I found it necessary to use a variety of approaches to sample dugongs (figure 3.1). The option of using dugong faeces as a DNA source has not previously been considered for marine mammals, and in future may replace the other more invasive methods. At present, the biopsy device is superior in the quality of sample provided and is the most efficient means of obtaining a sample from a free ranging dugong.

3.9 POTENTIAL SAMPLING ARTEFACTS

An ideal sampling program would not be limited by time or funds, and would enable the species to be randomly sampled across its range. The geographic pattern of sample localities and sample size depends on the scale of genetic substructure, which may not be apparent until the first round of sampling (Baverstock & Moritz, 1996). Therefore the time and financial budget of the study should consider the potential of additional rounds of sampling (Baverstock & Moritz, 1996). When using a (statistical) resampling approach, Weir (1996) recommends that 20 individuals from at least five localities be sampled.

I was unable to achieve such a sampling regime in this study. Because the dugong has a vast range, and mostly occurs in remote areas at relatively low densities in Australia, I used various sampling approaches and relied on cooperation from a network of collectors (Appendix A). Collection was essentially opportunistic, even when biopsy sampling free ranging dugongs (section 3.8). For this reason, the data set may not meet the random-sample requirement of some statistical methods.

As mitochondria are clonally inherited, individuals from one location are likely to be very similar in sequence. Often more than one individual

dugong is represented by the same haplotype (Chapter 7). Therefore with respect to statistical interpretation, the preferred initial sampling strategy is to collect a few individuals from many localities, rather than many individuals from a few localities. This study used such a sampling approach.

I have assumed individual resampling has not occurred. However, as resampling cannot be detected from the genetic marker employed in this study (as more than one individual can share a haplotype), the possibility that it has occurred cannot be excluded. C.J. Limpus (personal communication), who collected many of the biopsy samples, considered that resampling was unlikely. If resampling has occurred, the effect would be reflected in the haplotype frequencies, in a manner directly proportional to the level of resampling. The high occurrence of one gene type in Moreton Bay (Chapter 7) may indicate resampling. However, as this population had a number of samples collected from carcasses also with the same haplotype (Appendix C), I conclude that the observed haplotype frequencies are not an artefact of resampling free ranging dugongs.

The appropriate sample size is directly dependent on the variability of the genetic marker used. Greater genetic variability demands a larger sample size to account for individual variation. Hypervariable region 1 is one of the most variable regions in mtDNA (Chapter 6). With many rare haplotypes and a small sample size, there is an increased risk of making a type I error; that is, concluding that the intraspecific units are different when they are not. If the sampled localities are not characterised by rare haplotypes, but are differentiated by haplotype frequencies, then differences may not be detected and the probability of making a type II error increases. As populations of a significant sample size (20 individuals) showed an appropriate range of both rare and common haplotypes, a sample size of at least 10 and preferably 20 individuals per population was considered as 'ideal'. The DNA sequence divergence of haplotypes as well as haplotype frequency can be used to recognise intraspecific units. Therefore, a sample size of less than 20 is informative with respect to recognising intraspecific subdivision, even if it is less than ideal. I did not achieve a sample size of 20 for all the dugong populations. Of the 20 populations analysed in this study, 15 are represented by less than five individuals. Populations from remote areas with difficult access are represented by the fewest samples. However a sample size of one still provides some information as illustrated by the

interpretation of the sequence data in Chapters 7 and 8 (see figure 3.2 for populations and their respective sample sizes).

My statistical analyses may have missed subtle changes in haplotype frequencies which may define additional intraspecific subdivision other than that recognised by this study (type II error). Computer simulations have shown that when mutation rates are high (i.e., haplotype diversity approximates 'one') and sample size is small, sequence based statistics are more powerful than statistics based only on allele frequency (Hudson *et al.*, 1992). Baverstock & Moritz (1996) stated that the power of statistical tests for intraspecific subdivision may be greater when they incorporate information on molecular differences (DNA sequence) as well as haplotype frequency (e.g., Excoffier *et al.*, 1992).

I used statistical methods which employed both sequence information and haplotype frequency, because the evolution in the mtDNA control region is rapid and the sample size I obtained from each dugong population was usually small. Despite the limitations of the small sample sizes, the geographical specificity of haplotype sequences and haplotype frequencies provided an extremely informative and descriptive source of information to recognise intraspecific subdivision.

3.10 POPULATIONS REPRESENTED IN THIS STUDY

Of the 254 dugong specimens available (Appendix C), 103 dugong samples and one Florida manatee sample were sequenced (section 4.4). Not all of the samples collected were analysed. This was either because of the low sample quality (as discussed in section 3.1), or because the minimum sample size for a region (20 individuals, e.g., Torres Strait) had been met. The sample size and each locality (population) of the samples analysed (i.e., sequenced) in this study are illustrated in figure 3.2.

CHAPTER FOUR

MOLECULAR METHODS

In this chapter I outline the methods used to obtain the genetic data. Flow diagrams showing the steps involved to employ these molecular markers are provided. Two genetic markers were employed: mitochondrial DNA (mtDNA) and microsatellites. The cytochrome *b* gene and hypervariable region 1 (HVR-1) of the control region of mtDNA were explored.

4.1 EXTRACTION AND PURIFICATION OF DNA

4.1.1 DNA extraction from tissue

DNA was extracted from dugong and manatee tissue following a modification of the method of Lovell-Badge (1987). Approximately one gram of tissue, which had been minced with a sterile blade, was suspended in 2 ml of lysis buffer (40 mM Tris; 2 mM EDTA; 0.2 M NaCl; 10% SDS) contained in 10 ml polypropylene centrifuge tubes (Disposable Products). In order to break down most of the protein, the tissue was digested at 37°C with the proteolytic enzyme Proteinase K, which is active against a variety of indigenous proteins. Proteinase K (20 mg/ml) was added in 50 µl aliquots until there was complete digestion of the tissue, that is, until the lysate was clear, or almost so. The samples were stored at 4°C until the next step, phenol/chloroform extraction.

4.1.2 Removal of protein with organic solvents

Further deproteinisation was achieved by extraction with the organic solvents phenol and chloroform:isoamyl alcohol (24:1) (for preparation of these organic solvents, see Sambrook *et al.*, 1989). Standard procedure involved one or two (depending on the amount of protein debris at the interface) extractions with an equal volume of phenol, followed by one extraction with an equal volume of phenol-chloroform:isoamyl alcohol (24:1) to remove the protein, and one extraction of equal volume with chloroform:isoamyl alcohol (24:1) to remove the phenol (Sambrook *et al.*, 1989). During each extraction, the contents of the tubes were mixed by gentle rocking and then centrifuged at room temperature with a swing out

rotor in an International Clinical (bench) Centrifuge (model CL) at 2500 rpm for 15 minutes (min) or for smaller volumes (i.e., less than 1 ml), in either a Beckman microfuge E™ or an Eppendorf centrifuge 5415. The aqueous supernatant was aspirated, care being taken to leave behind the protein layer at the interphase.

4.1.3 Precipitation of the DNA and removal of RNA

Ethanol precipitation was used to concentrate DNA after enzymic reactions and to remove salts or reaction products following phenol and chloroform extractions (Crouse & Amorese, 1989). To precipitate DNA isolated from tissue, 0.1 volume of 3 M sodium acetate (pH 4.8) and 5 volumes of chilled absolute EtOH (AR grade) were added and the sample precipitated at -20°C ≥ 1 hour or at -70°C for 30 min. For cloned product precipitation, 0.02 volumes of 7.5 M ammonium acetate (pH 7.5) and 6 volumes of chilled absolute EtOH (AR grade) were added, and the solution chilled as above. The precipitate was concentrated in a microfuge at top speed (i.e., 14,000 rpm) for 15 min, the supernatant decanted and the pellet washed in 70% ethanol. A 'wash' involved mixing the solution by tube inversion followed by centrifugation, and decanting of the supernatant. An alcohol wash removed residual salt and facilitated dehydration of the pellet.

To remove RNA from DNA preparations such as isolated genomic DNA and cloned DNA (after mini preps, section 4.5.6), the dry pellets (from the precipitation step) were resuspended in storage buffer (TE: 10 mM Tris.HCl (pH 8.0); 1 mM EDTA (pH 8.0)) and DNase free RNase (Sigma) (10 mg/ml) were added to a final concentration of 20 µg/ml, followed by a 30 min incubation at room temperature (RT).

4.1.4 Purification of Polymerase Chain Reaction products

Polymerase Chain Reaction (PCR) products were purified using Magic PCR Preps (Promega) following the manufacturer's instructions. The PCR products were eluted with 100 µl of H_2O and an aliquot of the elution was run on an agarose check gel (section 4.2.2) in order to estimate the quality and quantity of the DNA.

4.1.5 Isolation of DNA from low melting point agarose gels

Low melting point agarose gels were used to purify and isolate DNA according to its size. The preparation of a low melting point agarose gel is outlined in section 4.2.2. Caution was employed when running low melting point agarose gels so they did not overheat.

The DNA sample was loaded alongside a size and concentration standard on a EtBr stained 1-2% low melting point agarose gel (Schwarz Mann Biotech) in 1 x TBE. The gel was photographed and the band(s) of interest, or the smear of DNA in the size range required (a smear represented DNA in a variety of sized fragments), were excised from the gel and transferred to a clean disposable microfuge tube. The DNA was then extracted from the low melting point agarose after heating at 60°C until melted. To purify the DNA, Magic PCR Preps (section 4.1.4), or phenol/chloroform extraction followed by precipitation of the DNA (sections 4.1.2 & 4.1.3 respectively) were used.

4.2 QUANTITATIVE AND QUALITATIVE ESTIMATION OF DNA

Two methods are widely used to measure the amount of nucleic acid in a preparation. If the sample is pure (i.e., without significant amounts of contaminants such as proteins, phenol, agarose, or other nucleic acids), spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the DNA is simple and accurate. If the amount of DNA is very small (i.e., <250 ng/ml) or if the sample contains significant quantities of impurities or degraded nucleic acids, as little as 10 ng of DNA can be quantified by comparing the fluorescent intensity of an EtBr stained sample DNA against a known concentration DNA standard run alongside on an agarose gel (Sambrook *et al.*, 1989). My choice of method was dependent on the size and status of the sample.

4.2.1 Spectrophotometric determination of DNA concentration

This method is routine for estimating the concentration of stock solutions of primers. To measure the amount of DNA, readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample. An Optical Density (OD) unit of one corresponded to approximately 50 µg/ml for

double stranded DNA and about 33 µg/ml for single stranded DNA (Titus, 1991). The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} values of about 1.8. If there was contamination with protein or phenol, the OD_{260}/OD_{280} would be significantly less than the values given above, and accurate measurement of the amount of nucleic acid was not possible (Sambrook *et al.*, 1989).

4.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used for qualitative and quantitative analysis of DNA. Factors influencing the migration rate of DNA fragments during electrophoresis are summarised by Sambrook *et al.* (1989).

Gels were prepared with agarose (Promega, molecular biology grade) in 1 x Tris-Borate buffer (TBE: 89 mM Tris, 89 mM Boric acid, 2 mM EDTA (pH 8.5)). The gel in TBE buffer was contained in a wide-based item of glassware, such as a beaker with a watch glass as a lid (to minimise evaporation) and heated in a microwave to dissolve. EtBr was added to a final concentration of 0.05 µg/ml and the gel poured when the beaker was cool enough to handle. The gel was allowed to set either at RT or in the fridge. The gels were run submerged in a horizontal electrophoresis apparatus (Pharmacia GNA-100 or Biorad wide Mini-sub Cell as appropriate) in TBE buffer (as above) at, for example, 60 mA for a 0.6% gel. Samples were co-loaded with approximately 0.2 volumes of tracker dye (0.25% Bromophenol blue; 40% Sucrose). In order to estimate concentration and size of the samples, known molecular weight and concentration standards (e.g., 100 base pair ladder (Pharmacia) and pUC18 respectively), were run along side the sample DNA. The DNA was visualised using EtBr which intercalated with the DNA and fluoresced when viewed under ultraviolet light on a 2011 Macrovue Transilluminator (LKB Bromma). A record was obtained by photographing the gel with a Direct Screen instant Camera (DS34, Polaroid) using Polaroid 667 film. Because the amount of fluorescence was proportional to the total mass of DNA, the quantity of DNA in the sample was estimated by comparing the fluorescent yield of the sample against the standard (Sambrook *et al.*, 1989).

4.3 THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) (Saiki *et al.*, 1988) is a revolutionary technique in molecular biology which can specifically amplify DNA from minute amounts of template DNA or tissue from a variety of sources. The other advantage of using the PCR, is that the product is suitable for direct sequencing (section 4.4), bypassing the time consuming and cumbersome step of cloning (section 4.5).

The PCR is based on the use of two oligonucleotides to prime DNA synthesis from the opposite strands across an intervening region. By repeated cycles of denaturation of the DNA strands, annealing of fresh oligonucleotide primers, and DNA synthesis, an exponential increase of a specific DNA fragment can be achieved (figure 4.1). The reaction is capable of generating microgram amounts of target DNA starting from a single copy.

4.3.1 Primer design

Primers are short sections of single stranded DNA (oligonucleotides) which have been designed to amplify DNA of a predicted size and region. Guidelines suggested by Hoelzel (1992) were considered when designing primers for this study. Primers should be of 17 to 24 bases in length, with 40-60% of GC content and matching annealing temperatures. Similarity and identity with the template should be 100% at the 3' end of the primer, while some differences are permitted at the 5' end. To avoid primer dimer formation, complementary sequence between primer pairs should be avoided and the potential for the primer to fold and anneal to itself especially at the 3' end, taken into account. These factors reduce the amount of primer available for the template. Higher annealing temperatures of the primers are preferred as the primers are less likely to anneal to non specific template DNA. The following equation was used to calculate the expected annealing temperature of the primer with no mismatch:

Annealing temperature (°C) = Melting temperature (°C) - 5 (°C).

Where the melting temperature (°C) = 2 (total AT bases) + 4 (total GC bases). Other factors which can be considered in primer design are discussed by Hoelzel (1992).

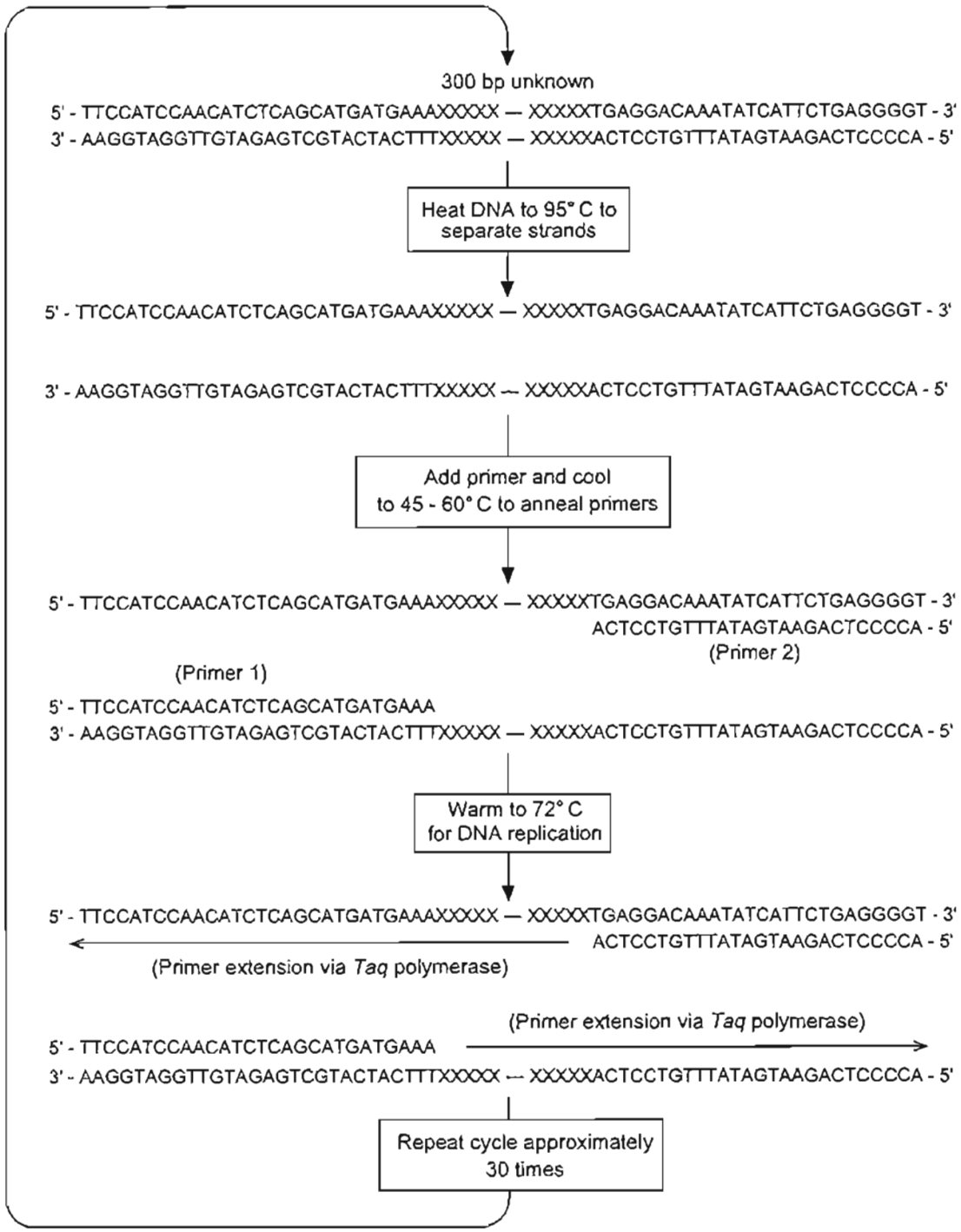


Figure 4.1: The Polymerase Chain Reaction (PCR). An exponential increase of a specific DNA fragment can be obtained by repeated cycles of DNA denaturation, annealing of primers, and DNA synthesis (extension).

Primers were designed to amplify segments of the mtDNA control region and cytochrome *b* gene, and microsatellite loci. To sequence the entire mtDNA control region, I initially used the control region primers designed by Kocher *et al.* (1989) to amplify the entire control region in the dugong (about 2,000 bases long), of which the sequence was obtained in a 'stepwise' fashion as only 200 to 300 bases could be sequenced at one time. The 5' and 3' ends of the control region PCR product were sequenced, followed by sequential design of dugong specific primers, amplification and sequencing of the product, until the complete sequence of this region was obtained. Primers designed are listed in table 4.1.

4.3.2 Primer purification

Primers manufactured within the Department of Biochemistry (James Cook University of North Queensland) were 'deprotected' before I received them. As the primers were received contained in ammonia, they were divided into 300 µl aliquots and the ammonia evaporated using a DNA Speed Vac (Savant) in a fume hood for approximately 3 hours at 43°C. That is, until the pellet was completely dried. 50 µl of 80% acetic acid was added to the residue and allowed to stand at RT for 30 min. 1 ml of diethyl ether was added, mixed well and the mix allowed to stand at RT for 15 min. The primer was pelleted in a microfuge for 10 min, the supernatant decanted (carefully so the pellet was not dislodged) and air dried. If the pellet was glutinous, it was rewashed in 1 ml diethyl ether, re-centrifuged and dried. The diethyl ether wash was repeated until the pellet became powdery. Then the pellet (of primer) was resuspended in 200 µl of H_2O and the purity checked following the protocol in section 4.2.1.

4.3.3 The Polymerase Chain Reaction

Amplification was performed either in heat sealed microsyringe tips (Bresatec) in a Corbett Research FTS-1 Thermal Cycler for reactions totalling a volume of 20 µl; or in 0.5 µl microfuge tubes (Quantum Scientific) in a Hybaid Thermal Cycler for 100 µl reactions. In a standard PCR reaction I used 10 pmol of each primer; 1 x buffer (10 x Mg free dilution buffer was supplied with the DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) (Promega); 1.75 mM of each dNTP; 2.5 mM $MgCl_2$ and up to 20 ng of genomic DNA. Optimal PCR conditions were resolved using 20 µl reactions, which were often repeated as a 100 µl reaction to produce the amount of template required for direct sequencing.

Table 4.1: Oligonucleotides used.

Region	Oligo	T_m	Sequence	ref.
pUC primer 40 bases downstream from polylinker	-40 USP	52	5'-GTTTTCCCAGTCACGAC-3'	A
pUC primer 40 bases upstream from polylinker	-40 RSP	58	5'-AGCGGATAACAATTTCACACA-3'	B
mtDNA control region (L15926)*†	A24 (L)	76	5'-TCAAAGCTTACACCAGTCTTGTAAACC-3'	C
mtDNA control region (L16007)*†	A25 (L)	50	5'-CCCAAAGCTAAAATTCTAA-3'	C
mtDNA control region (H00651)*†	A26 (H)	78	5'-TAACTGCAGAAGGCTAGGACCAAACCT-3'	C
d.s. mtDNA control region, internal†	A55 (L)	58	5'-CCACACCATAGGATATTGC-3'	D
d.s. mtDNA control region, internal†	A56 (H)	75	5'-CCAGCGGGGTCCGTTTCGATCC-3'	D
d.s. mtDNA control region, internal†	A77 (L)	62	5'-CGAGAAACCAGCAACCCGC-3'	D
d.s. mtDNA control region, conserved block†	A57 (L)	66	5'-GACATCTGGTTCC/TTACTTCAGG-3'	D
d.s. mtDNA control region, conserved block†	A58 (H)	66	5'-CCTGAAGTAG/AGAACCAGATGTC-3'	D
d.s. mtDNA control region, repeat†	A78 (L)	56	5'-CAGTCAATGCTCGAGGAC-3'	D
d.s. mtDNA control region, repeat†	A80 (H)	58	5'-CCCGTACCCTTACTTTCTG-3'	D
d.s. mtDNA control region, central†	OM1 (L)	54	5'-TCCACACCATGGATATTGT-3'	D
d.s. mtDNA control region, central†	OM2 (H)	60	5'-TAAGCCTCAGACGGCCTAG-3'	D
d.s. mtDNA, cyt *b* to control region†	DT1 (L)	68	5'-GCTACTGCCTTCATAGGGTACG-3'	D
d.s. mtDNA, cyt *b* to control region†	DT2 (H)	68	5'-CCTCTTCCCTGAATATCCTCGG-3'	D
mtDNA cytochrome *b* (L14841)*	DB1	96	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'	C
mtDNA cytochrome *b* (H15149)*	DB2	98	5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'	C
Dugong microsatellite	MS1	58	5'-CAGCAGTTGCAATCCACCA-3'	D
Dugong microsatellite	MS2	58	5'-CCAGCTCTTAACCACTGTG-3'	D
Dinucleotide-microsatellite probe	$(GA)_{10}$	n.a.	5'-GAGAGAGAGAGAGAGAGAGA-3'	E
Dinucleotide-microsatellite probe	$(GT)_{10}$	n.a.	5'-GTGTGTGTGTGTGTGTGT-3'	E
Trinucleotide-microsatellite probe	$(CGG)_{10}$	n.a.	5'-CGGCGGCGGCGGCGGCGGCGGCGGCGG-3'	B
Trinucleotide-microsatellite probe	$(CAC)_{10}$	n.a.	5'-CACCACCACCACCACCACCACCACCAC-3'	B
Tetranucleotide-microsatellite probe	$(GATA)_7$	n.a.	5'-GATAGATAGATAGATAGATAGATAGATA-3'	B

Legend

d.s. = dugong specific

T_m = estimated melting point of the primer (°C).

n.a. = not applicable

L and H refer to Light and Heavy strands of mtDNA

* number (in brackets) refers to the position of the 3' primer in human mtDNA sequence (Anderson *et al.*, 1981).

† Refer to figure 6.1 for placement of control region primers.

ref. (references):

A Obtained from a commercial sequencing kit

B Supplied by the Biochemistry Dept, James Cook Uni. of N.Qld

C Designed by Kocher *et al.*, (1989)

D Designed by D. Tikel (author)

E Supplied by A. Brooker, AIMS, Townsville. N.Qld

The contents of the reaction tubes were flick mixed followed by brief centrifugation to settle the contents. The mixes were then incubated at 95°C for 2 min to denature the double stranded DNA and then chilled on ice to anneal the template-primers. 1 unit (U) of *Taq* polymerase was added, flick mixed and the contents settled by centrifugation. For the 20 µl reactions, a plug to prevent evaporation is provided as part of the microsyringe. With 100 µl reactions, evaporation was prevented by the addition of 65 µl of mineral oil. Table 4.2 details the programs for standard PCR cycles for the Corbett Research FTS-1 Thermal Cycler and Hybaid Thermal Cycler.

Table 4.2: Standard PCR cycling programs. Prior to running samples in these programs, the reactions were incubated for 2 min at 95°C and then chilled on ice, then the *Taq* polymerase was added.

Program for 20 µl reactions run in a Corbett Research FTS-1 Thermal Cycler:

Cycle	Step	Temperature °C	Time	Times this cycle
1	1	94	35 sec	1
	2	45	45 sec	
	3	72	1 min 30 sec	
2	1	95	20 sec	29
	2	45	5 sec	
	3	55	10 sec	
	4	72	1 min 30 sec	
3	1	72	5 min	1

Program for 100 µl reactions run in a Hybaid Thermal Cycler:

Cycle	Step	Temperature °C	Time	Times this cycle
1	1	96	35 sec	30
	2	45	10 sec	
	3	55	30 sec	
	4	72	1 min 30 sec	
2	1	72	5 min	1

Optimisation of the PCR was a process of 'trial and error' experimentation. Primarily the activity of *Taq* polymerase was checked. Then fresh dilutions of the primer and/or template, and new stocks of the reagents: (10x) buffer, H_2O, dNTP's, were used. The PCR program was altered, usually by lowering annealing temperatures. The template/primer ratio was altered, and various $MgCl_2$ concentrations were tried. 0.2% (final concentration) of BSA was added to the PCR reaction to 'mop up' contaminants. In addition, a PCR optimisation kit (Boehringer Mannheim) was employed to optimise reactions. It has (amongst other optimising reagents) a range of buffers containing various Mg concentrations and pH. This kit proved invaluable for optimising PCR conditions.

Negative (PCR without DNA) and positive (PCR with DNA) controls were used in the following situations:

- During PCR optimisation.
- When new reagents were introduced.
- If contamination was suspected.
- To test reagents suspected of having lost their activity.

If non specific PCR products were produced, the band of interest (identified by comparing against a size standard and the fact that it was in greater concentration (stained brighter with EtBr)) was excised after running the product on a low melting point agarose gel, purified using Magic PCR Preps (see section 4.1.4) and then re-amplified. If the PCR product was in sufficient concentration, it was used directly in sequencing.

4.4 DIRECT vs INDIRECT SEQUENCING

There are two approaches to prepare templates for sequencing, direct and indirect (figure 4.2). Both methods involve an initial PCR (section 4.3.3) to amplify a double stranded DNA segment of interest to very high copy numbers.

Direct sequencing has the following advantages over conventional cloning (indirect sequencing) of PCR fragments into plasmids or viral genomes: 1) it eliminates the need to clone the DNA segment to be sequenced, so there is no tedious and time consuming technique involved as with cloning; 2) since it is an *in vitro* system that does not depend on living organisms

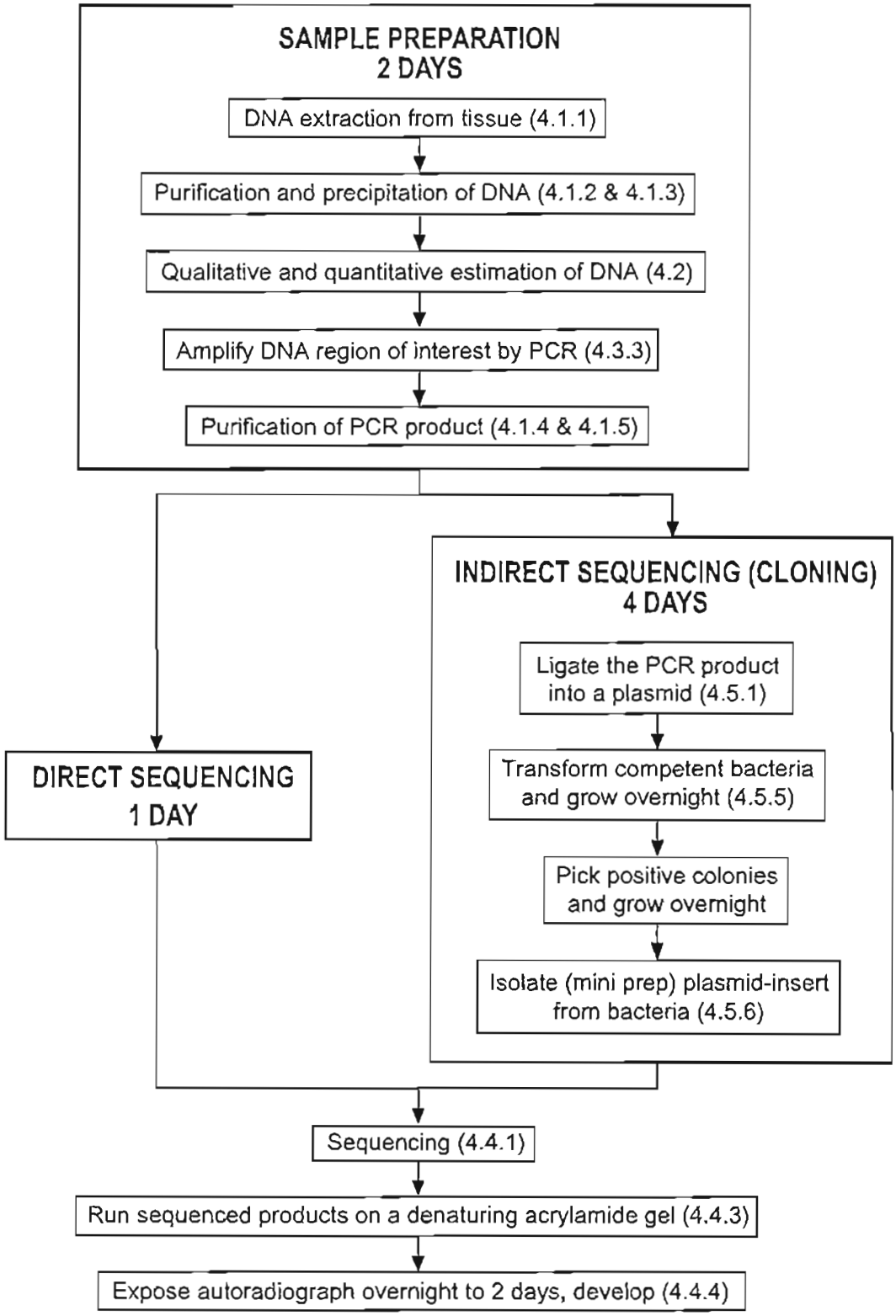


Figure 4.2: The direct and indirect approaches to sequencing. The numbers in brackets refer to the relevant sections within this chapter.

(bacteria, virus) it can be more readily standardised, thus subjected to automation, and 3) as it is considered more reliable, only a single sequence needs to be determined for each sample. With respect to cloned PCR products, two or three clones should be sequenced for each sample in order to exclude: a) mutations due to random misincorporated nucleotides (produced by DNA polymerase during PCR) and, b) artefacts of the amplification such as the formation of mosaic alleles ('shuffle clones') by *in vitro* recombination (Gyllensten, 1989). Initially, I tried to obtain the sequence of interest via direct sequencing for the reasons discussed. If this was not successful, I resorted to cloning the fragment of interest followed by sequencing.

4.4.1 Sequencing

DNA was sequenced using the method devised by Sanger *et al.* (1977b) as illustrated in figure 4.3. Both PCR product and cloned product were treated the same way in preparation for sequencing, and in the sequencing reaction itself. However, often more PCR product than cloned product was required to produce a successful sequencing reaction.

A variety of sequencing (commercial) kits were tried. The most successful sequencing systems/kits were employed on a regular basis. This included: Amersham's Multiwell microtitre plate DNA sequencing system (T7 DNA polymerase), Pharmacia's Sequenase T7 DNA polymerase and, USB's T7 Sequencing™ Kit. The sequencing reactions were carried out according to manufacturer's instructions, each kit varying slightly in its protocol. All reactions required the incorporation of a radioactive marker. Phosphorus 32 (P^{32}) was the radioactive isotope routinely employed.

4.4.2 Denaturation of double-stranded template before sequencing

Both PCR and cloned templates were denatured in preparation for sequencing following a denaturation protocol modified from Amersham's Multiwell microtitre plate DNA sequencing system (T7 DNA polymerase). Approximately 5 µg of the double stranded template (PCR or cloned product) was added to 8 µl of 2 M NaOH/2 mM EDTA and H_2O was added to a final volume of 80 µl. The tube was vortexed briefly, centrifuged to settle its contents and incubated at 37°C for 15 min. The mixture was precipitated (section 4.1.3) and the pellet was dried thoroughly before resuspension in

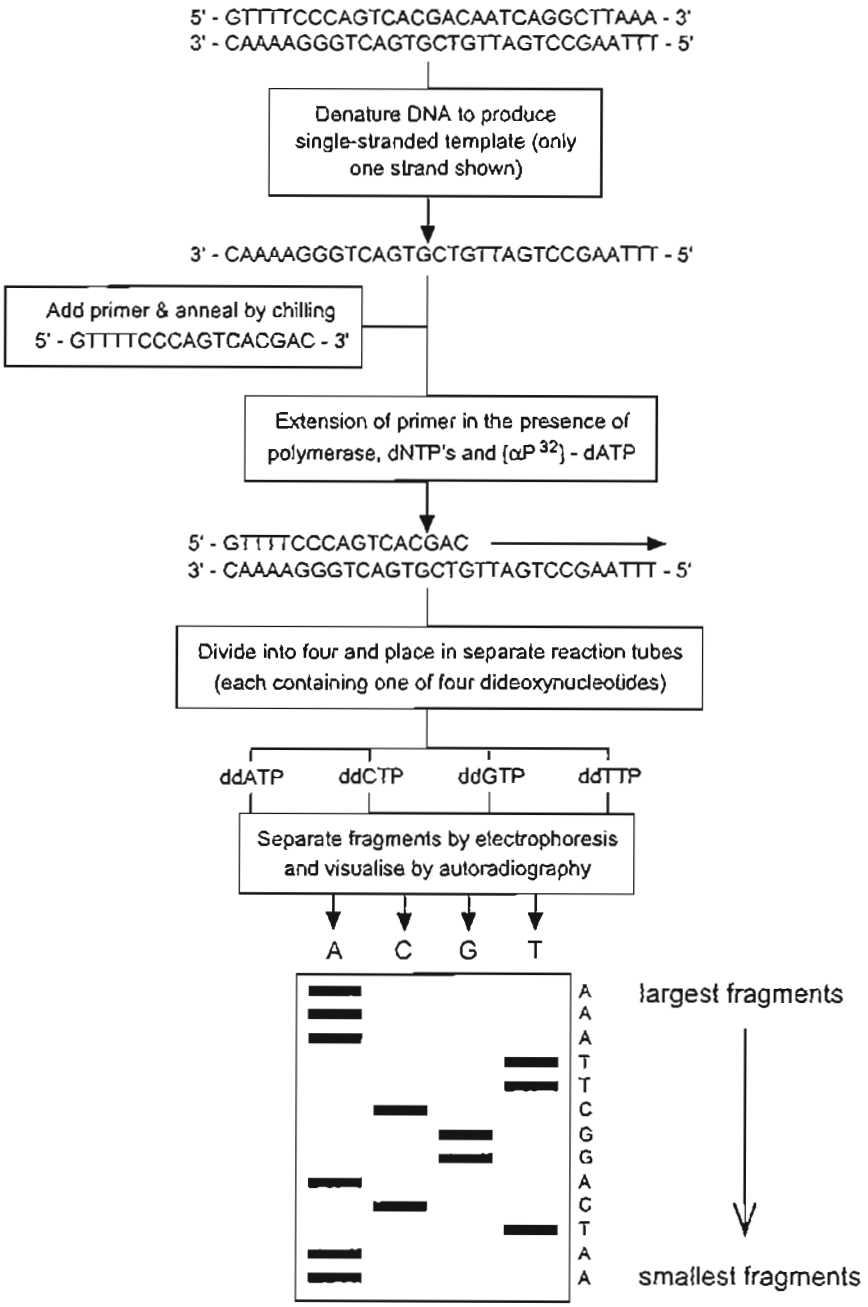


Figure 4.3: The DNA sequencing method developed by Sanger *et al.* (1977b). After a labelling reaction where the marker (radioactive isotope) is incorporated into copies of the template, the reaction is divided into four independent termination reactions. The incorporation of a dideoxynucleotide stops further extension as these cannot form a phosphodiester bond with the next incoming nucleotide. The products of each reaction are a series of incompletely extended segments, which are separated (one base pair apart) by acrylamide gel electrophoresis (Hillis & Moritz, 1990; Watson, 1987).

the required volume for the sequencing reaction. I usually denatured the templates the day before sequencing and inverted the tubes after precipitation, allowing the pellet to air dry O/N.

All of the pellet (denatured DNA) was used in a sequencing reaction with 10 pmol of primer. If the sequencing failed, a greater amount of template was tried. Successful direct sequencing was mostly determined by the cleanliness of the PCR product. If the sequencing failed to work after repurification of the PCR product (section 4.1.4), the PCR product was cloned and then sequenced. Each primer of a pair used for PCR was trialed as I found that some primers sequenced better than others. Sequencing products were run on 6% to 8% denaturing acrylamide gels as described in the following section.

4.4.3 Denaturing acrylamide gels

Denaturing acrylamide gels of 6% to 8% were prepared using either Acrylamide/Bis, Acrylamide/PDA or Sequagel acrylamide gel mix in 1 x TBE (the TBE in the upper well of the sequencing apparatus was recycled). The gels were prepared and run in a Biorad sequencing apparatus and the optimum temperature maintained at 55°C by adjusting the voltage (1600 to 2100 V). By co-loading the DNA sample (i.e., sequencing reaction) with a dye, the period of time for the samples to run through the gel could be predicted (Sambrook *et al.*, 1989). A sequencing run took about 2.5 hours (single run) to 6 hours (double run). After the run, the gels were washed for 15 min in 10% acetic acid to fix the gel and remove the urea (which otherwise prevents the gel from drying completely), rinsed in tap water to remove excess acetic acid, and transferred to a piece of Whatman 3MM paper. The acrylamide gel was then dried under vacuum for 1 hour at 88°C on a Biorad model 583 gel dryer. Once dried the gels were exposed O/N to autoradiograph film.

4.4.4 Autoradiography

In order to visualise and record the presence of the radioactive isotope P^{32}, Fuji autoradiograph film was placed against the acrylamide gel or Hybond N membrane (Amersham), which was then contained within an autoradiograph cassette. If the gel or membrane surface was moist, a layer of plastic wrap (i.e., Gladwrap) was placed at the interface. The strength of the radioactive signal was checked with a geiger counter (cpm) prior to

autoradiography. If the counts were low, a Dupont Cronex Lightning Plus intensifying screen or Amersham Hyperscreen (external to the autoradiograph film) was used, and the cassette placed at -70°C to assist amplification of the signal. Generally film was permitted to expose O/N (up to a week if the signal was very faint or if it was a re-exposure) and then developed (ILFORD Phenisol developer) and fixed (ILFORD Hypam rapid fixer) under safety light (Kodak GBX-2 safelight filter with a 75 watt clear globe) for about 3 min each. After fixing, the film was rinsed in water and allowed to drip dry.

4.5 CLONING

Cloning is the traditional or indirect means of producing large quantities of a specific DNA fragment. The homogenous product can subsequently be sequenced, exposed to restriction endonuclease digestion and/or used as a probe. The technique involves transformation of bacterial cells with a DNA segment of interest inserted in a circular recombinant plasmid (vector) which contains the gene for antibiotic (ampicillin) resistance as well as an origin of autonomous DNA replication. The bacterial cells produce copies of plasmid-insert at the same time as they replicate. The plasmid-insert DNA, enriched by the bacteria, can then be selectively extracted and subjected to sequencing. Although the general method of cloning is the same, slightly different ligation procedures (i.e., different vectors) and more than one type of cell line were employed, according to what was available and/or appropriate at the time.

4.5.1 Ligation

Two ligation approaches were used to clone PCR and restriction enzyme products. Following manufacturer's instructions, the pCR™ Vector (TA Cloning kit produced by Invitrogen) was used to ligate PCR products. Alternatively, PCR products and 'blunt-ended' restriction enzyme digested DNA, were blunt-end ligated into *Sma*I cut, dephosphorylated plasmid, pUC18, as detailed in the following sections.

4.5.2 Blunt-ending the PCR product

As *Taq* polymerase has a tendency to add an adenine overhang, the PCR product had to be blunt-ended before ligation to a blunt-ended vector. The PCR product was run on a low melting point agarose gel (section 4.1.5), the specific band excised and the DNA extracted using the Magic PCR preps (section 4.1.4). Up to 1 µg of the eluted PCR product, 1 x final concentration of Nick Translation buffer (10 x stock = 0.5 M Tris, 0.1 M $MgSO_4$, 10 mM DDT, 500 µg/ml BSA), 0.2 mM final concentration of dNTP, 1 mM final concentration of ATP, 10 U of T4 Polynucleotide Kinase (Promega), 10 U of DNA polymerase 1, and H_2O to a final volume of 50 µl, were mixed in a 1.5 µl microfuge tube. The blunt ending reaction mix was incubated at 37°C for an hour and then stopped with 1 µl of 0.5 M EDTA (pH 8.0).

Following the blunt-ending, the DNA was cleaned following the method for removal of protein with organic solvents (section 4.1.2) and precipitated using 0.5 volumes of 7.5 M ammonium acetate (pH 7.5-8.0) and 2 volumes of absolute EtOH (AR grade) and incubated at either -20°C for a minimum of 2 hours or at -70°C for 20 min. The samples were centrifuged for 15 min and the supernatant discarded. The pellet was washed in 500 µl of 0.67 M ammonium acetate/67% alcohol and centrifuged for 15 min and the supernatant discarded. A final wash with 90% ethanol was followed by centrifugation for 15 min and the supernatant discarded. The pellet was dehydrated and resuspended in 30 µl of H_2O. 1 µl was run on an agarose check gel (section 4.2.2) to estimate the concentration of the blunt-ended DNA.

4.5.3 Insertion into a plasmid vector

The ligation cocktail was composed of the appropriate amount of *Sma*I/BAP pUC18 (amount of vector added was dependent on the size and concentration of insert), 1 x T4 DNA ligase buffer, 50 µg/ml final concentration of BSA, 15% final concentration of PEG 8000 and 1 U of T4 DNA ligase (Promega) and the volume made up to 30 µl with H_2O. The ligation cocktail was flick-mixed and incubated O/N at 16°C . The ligation mix could be stored at this stage at -20°C until ready for transformation.

To check transformation efficiency, two controls were run at the same time as the experimental ligation: 1) 0.1 µg *Eco* R1 cut pUC18 and, 2) 0.05 mg of uncut pUC18.

4.5.4 Preparation of competent cells

Using the manufacturer's instructions of the Bresa M13 Cloning Kit (MCK-18), 50 mls of sterile Luria Broth (LB) medium (1% Bacto-Tryptone; 0.5% Bacto-Yeast Extract; 1% NaCl) were inoculated with 1 to 2 ml of the *Escherichia coli* bacterial strains JM101 or NM522, and incubated on a shaking incubator (at 300 oscillations per minute) at 37°C until the bacterial growth had reached mid-log phase (i.e., the OD_{600} was between 0.45 and 0.55). The bacteria were placed on ice for 30 min, pelleted at 2500 g for 10 min at 4°C in a fixed angle rotor of a Sorvall centrifuge (Superspeed, RC2 automated refrigerated centrifuge), the supernatant decanted, and the pellet resuspended in 25 ml of 0.1 M $MgSO_4$, left on ice for 40 min, and centrifuged as before. The pellet was resuspended in 5 ml of ice cold 0.1 M $CaCl_2$. Incubation of logarithmic growth phase *E. coli* cells makes them permeable to DNA (competent) by incubation in $CaCl_2$. The JM101 cell line can be stored on ice from 12 to 20 hours before transformation, whereas the NM522 cell line is most competent if used immediately after treatment. The NM522 cell line was preferred as its transformation efficiency was higher.

4.5.5 Transformation of competent cells

A small plasmid, pUC18 (2686 bases), has an ampicillin (Amp) resistance gene and a portion of the *lac* z gene of *E.coli*. Within the *lac* region is a polylinker sequence of unique restriction enzyme recognition sites (multiple cloning site). When a plasmid (pUC18) without an insert is transformed into a *lac-E.coli* and grown in the presence of IPTG, a *lac* operon inducer, the enzyme galactosidase is produced. These cells hydrolyse the galactose analogue X-gal giving rise to blue colonies. When DNA fragments are inserted into the multiple cloning site of pUC18, the *lac* gene is inactivated. When these insert-plasmids are transformed into the same cells, the production of galactosidase is interrupted giving rise to colourless colonies. These transformants are selected from unsuccessfully transformed cells by plating the bacterial cells onto LB-agar containing the antibiotic, Amp. The colourless colonies contain the insert-plasmid and are picked off and grown O/N as mini preps (Davis *et al.*, 1986).

The properties of competent cells and plasmids were exploited as follows: 10 µl of ligation mix was added to 200 µl of NM522 competent cells in $CaCl_2$ and stored on ice for 30 min. The efficiency of uptake of plasmid DNA from

the surrounding buffer was enhanced by brief heat shock treatment of the cells by incubating them at 3 min at 42°C and then placing them on ice for 10 min. 2 ml of SOC medium (2% Bacto-Tryptone; 0.5% Yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM each of $MgCl_2$ & $MgSO_4$; 20 mM Glucose) was added to the ligation mix and then incubated in a shaking incubator for 1 hour at 37°C. The transformed cells were centrifuged and resuspended in 600 µl LB. Just before plating the cells, 40 µl of X-gal (50 mg/ml in DMF) and 20 µl IPTG (100 mM) were spread on each plate. The transformed cells were then spread evenly over the plate maintaining sterile technique.

4.5.6 Mini preps of transformed cells

DNA was extracted from the bacterial cells using either Magic Mini Preps (Promega) following manufacturer's instructions, or by using the following Alkaline Lysis method modified from Sambrook *et al.* (1989).

The plasmid mini prep technique was used to prepare plasmid DNA in small quantities from a number of transformants. Isolation and purification is achieved by alkaline lysis to free the plasmid DNA from the cell, followed by removal of cell wall debris by centrifugation and selective alcohol precipitation to separate plasmid from chromosomal DNA. Separation is possible due to the different solubilities of small circular (plasmid) and large linear (chromosomal) DNA. The purity of the plasmid preparations is determined by agarose gel electrophoresis.

Several well isolated white colonies were selected from the plates containing Amp which had been incubated O/N, subcultured into 5 ml aliquots of LB containing Amp (50 µg/ml) and placed in the shaking incubator O/N at 37°C. 1.5 ml aliquots of the O/N culture were placed into 1.5 ml microfuge tubes and centrifuged at 5,500 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 150 µl of GTE solution (50 mM Glucose; 25 mM Tris-HCl (pH 8); 10 mM EDTA). 300 µl of lysis buffer (1% SDS; 200 mM NaOH) was added to each preparation and mixed by gentle inversion before being incubated on ice for 5 min. 225 µl of 3 M potassium acetate (pH 4.8) was added, the samples were mixed gently by inversion and incubated on ice for 5 min before being centrifuged at 14,000 rpm for 8 min. The supernatant was carefully aspirated and transferred to a new tube. One volume of chilled absolute EtOH (AR grade) was added, vortexed and then centrifuged at 14,000 rpm for 10 min to precipitate the

DNA. The pellets were then washed in 70% ethanol, the pellet dried and resuspended in 50 µl of storage TE containing RNase (section 4.1.3).

4.5.7 Preparing glycerol stocks

Bacteria can be stored indefinitely in cultures containing 15% to 50% glycerol (Sambrook *et al.*, 1989). Bacterial cells for storage were cultured by aseptically inoculating single colonies into 5 ml of LB medium containing 50 µg/ml Amp, and placed O/N at 37°C in the shaking incubator. 0.5 ml of 80% glycerol sterilised in the microwave for 2 min and cooled (alternatively sterilised by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle) was added to 1 ml of each culture contained in 2 ml Nalgene cryovials, and vortexed to ensure the glycerol was evenly dispersed. The cultures were initially frozen O/N at -20°C, and were then stored long-term at -70°C.

4.5.8 Recovery from glycerol stocks

The frozen surface of a culture was scraped with a sterile inoculation loop and the bacteria adhering to the loop were streaked onto the surface of LB agar plates containing Amp (50 µl/ml). The frozen culture was returned to -70°C. The plates were incubated at 37°C O/N (Sambrook *et al.*, 1989).

4.6 CONSTRUCTION AND SCREENING OF A SIZE-SELECTED LIBRARY FOR THE DETECTION OF MICROSATELLITE LOCI (figure 4.4).

Dugong DNA was isolated from fresh tissue (to ensure that high molecular weight DNA was obtained) of a dugong from Townsville (#328, see Appendix C for sample details) following section 4.1.1, and run on an agarose check gel (section 4.2.2) to check quality and quantity. Approximately 50 µg of this genomic DNA was digested with 100 U of both *Alu* I and *Rsa* I (Pharmacia) restriction enzymes. Both of these restriction enzymes are blunt-end cutters, and in combination, are expected to cut genomic DNA at the frequency to produce a small-insert (100's bp) library. The digested DNA was run on an 1% low melting point agarose gel (section 4.1.5) alongside a 100 base pair size marker (Pharmacia) and fragments of the size range 200 to 600 bp were excised from the gel using a sterile blade. The DNA was isolated from the gel using Magic PCR preps (section 4.1.4) with a final eluate volume from the purification column of

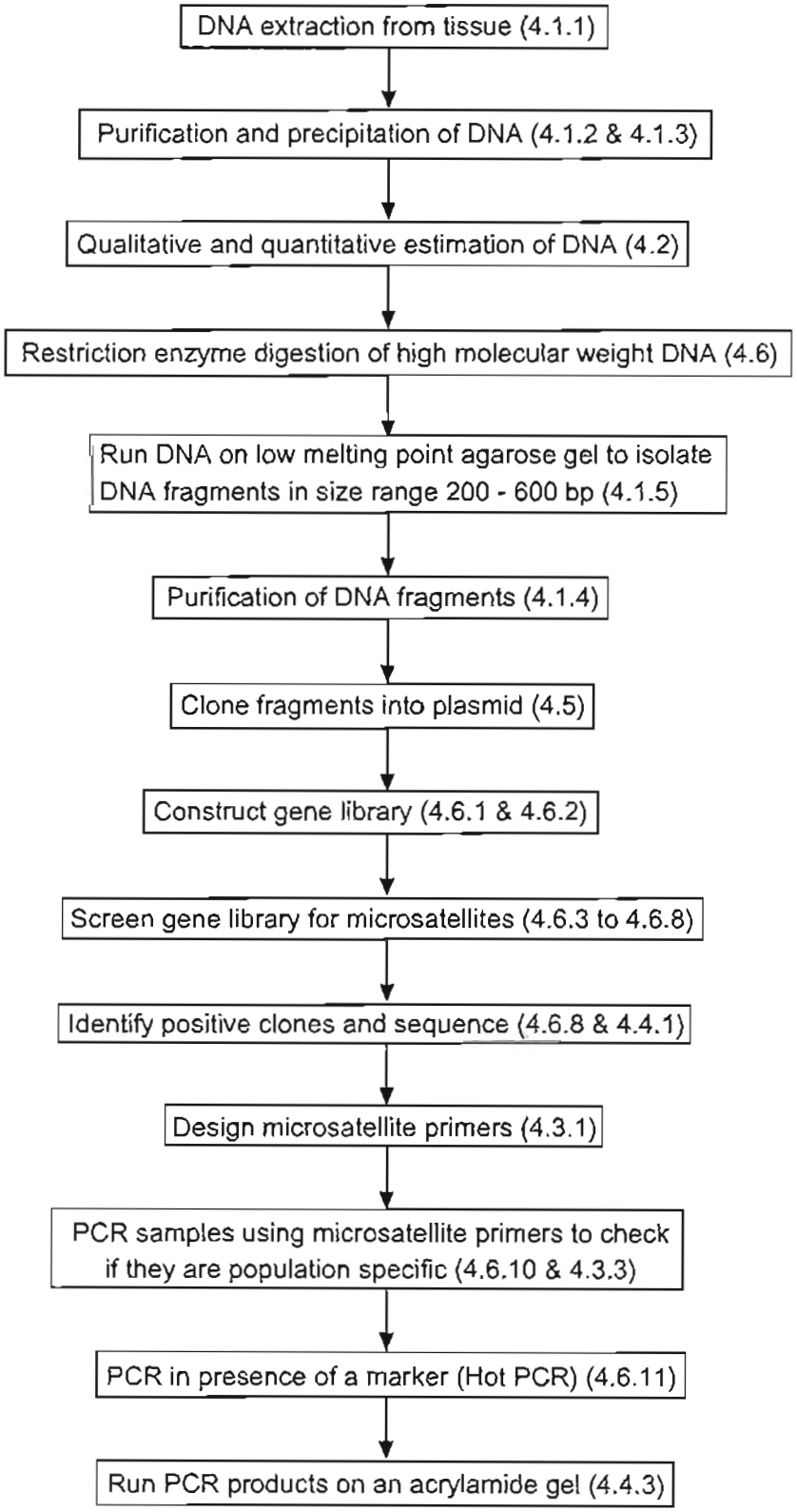


Figure 4.4: **The methods employed for developing microsatellite markers.** The relevant sections in the chapter are indicated within brackets.

100 µl H_2O. The concentration and efficiency of extraction was checked on a 0.6% agarose gel (section 4.2.2) before ligation (section 4.5.1) and transformation (section 4.5.5).

4.6.1 Colony blots

Three gridded plates are prepared for colony blots, one master plate to which the colony is 'dotted' onto the agar with no membrane, and two replicate plates where the colony is scratched onto a gridded membrane placed on top of the agar. The master plates function as a short-term store for each colony, and the grids enable identification of each colony among the replicates.

Grids were drawn upon Hybond N (Amersham) membranes using a 2B (soft leaded) pencil, labelled and the orientation indicated by clipping the top right hand side of the membrane. The membranes were laid upon an LB petri dish (plate) containing 100 µg/ml Amp until wet through. The grid pattern for the master plate comprised of a photocopy of a numbered grid, cut to the shape of the plate, and taped onto the base of the petri dish - the pattern is seen through the agar.

From plates containing transformed cells (section 4.5.5), positive (white) colonies, as well as one control (blue) colony, were 'dotted' onto a master LB plate using sterile toothpicks (one per colony). That same toothpick was 'scratched' onto the surface of a further two membranes with a matching grid pattern.

The petri dishes were incubated on their lids to avoid condensation on the agar at 37°C for 2 hours, and then at RT O/N wrapped in alfoil (to prevent dehydration and provide protection from light, as Amp is light sensitive). The master plates could be temporarily stored at 4°C (i.e., up to two weeks).

4.6.2 Lysis of colonies and binding of DNA to the Hybond N membrane

After the colonies were grown O/N, the colonies were fixed to the Hybond N membranes (Amersham) according to the manufacturer's instructions as follows. The membranes were placed onto a pad of 3MM Whatman paper, colony side up, which had been saturated in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 7 min. The membranes were then placed colony

side up on 3MM Whatman paper soaked in neutralisation solution (1.5 M NaCl; 0.5 M Tris-HCl (pH 7.2); 0.001 M EDTA) for 3 min. This was repeated with a fresh pad soaked in the same solution. The membranes were then rinsed in 2 x SSC (20 x stock: 3 M NaCl; 0.3 M Na_3Citrate) to remove the cellular debris. The filters were then blotted, colony side up, on dry 3MM Whatman paper and allowed to air dry for at least 30 min.

To fix the DNA to the membrane, the membranes were oven baked at 80°C for 2 hours. Membranes were stored after this step by sandwiching them between 3MM Whatman paper, heat sealed in plastic and stored at 4°C.

4.6.3 End labelling oligonucleotides

Oligonucleotides (primers and repeat oligos) were 5' end-labelled using a Ready-To-Go™ T4 Polynucleotide Kinase (T4PNK) kit following the manufacturer's instructions (Pharmacia). The lyophilised pellet (containing the T4PNK) was resuspended by pipetting up and down in 25 µl of H_2O, at RT for 2 to 5 min. 10 pmol of the oligonucleotide (to be labelled) was added along with sufficient H_2O to bring the final volume to 49 µl. 10 µCi of [γP^{32}] -dATP was added and the contents mixed gently. The contents were settled by brief centrifugation and incubated at 37°C for 45 min. The reaction was stopped by the addition of 5 µl of 0.25 M EDTA. This 'stop' was omitted when labelling primers in preparation for 'hot PCR' as I was concerned that the EDTA may inhibit the enzymatic efficiency of the *Taq* polymerase. The initial denaturation step of PCR would be expected to be sufficient to inhibit further activity of the T4PNK, hence acting as a substitute 'stop' for the reaction. Oligonucleotides end-labelled as probes and microsatellite primers are listed in table 4.1.

4.6.4 Prehybridisation of the membrane

While the probe was being end-labelled, the membranes were prehybridised. The membranes were arranged between mesh, and rolled following manufacturer's instructions such that they could be placed within the Hybrisation oven cylinders (Hybaid). Enough 2 x SSC was added to wet the membrane/mesh sandwich. When the sandwich was rolled, it was very important to ensure that there were no bubbles (which prevented the buffer and hence the probe, making contact with the membranes). The tightly rolled sandwich was placed into the cylinder containing 10 ml of 2 x SSC, taking care to place it in the correct orientation (see manufacturer's

instructions) to ensure that the direction in which the sandwich was rolled conformed with the direction in which the cylinder rotated in the hybridisation oven. Holding the lid on my left, I unrolled the sandwich by slowly turning the cylinder towards myself, the SSC was poured off and replaced with 15 ml of prewarmed Hybridisation buffer (5 x SSPE (20 x stock: 3.6 M NaCl; 0.2 M NaH_2PO_4; 0.02 M EDTA (pH 7.7)); 5 x Denhardt's solution (2% BSA; 2% Ficoll (Amersham); 2% PVP); 0.5% SDS) containing dissolved 0.5% Blotto (i.e., 0.075 g of skim milk powder). SSC was used to wet the membrane-mesh sandwich in preference to the Hybridisation buffer because the SDS in the Hybridisation buffer had a tendency to form bubbles (foam).

4.6.5 Hybridisation

The probe was denatured at 95°C for 5 min and added to the cylinder containing the membranes. The membranes were hybridised O/N at 55°C in a Micro-4 Hybridisation oven (Hybaid). In addition, the wash solutions were prepared and warmed to wash temperature O/N.

4.6.6 Washes following hybridisation

The membrane/mesh sandwich was unrolled in an glass pyrex dish and the membranes laid DNA side down in about 200 ml of 2 x SSPE, 0.1% SDS. The tray was agitated gently for 10 min at RT. The wash solution was removed and replaced with another 200 ml of 2 x SSPE, 0.1% SDS and gently agitated for 15 min at 60°C in a Grant's shaking water bath.

Incorporation was checked with a geiger counter. If the counts were very high, the lower salt concentration (higher stringency) wash was carried out. That is, approximately 200 ml of 0.1 x SSPE, 0.1% SDS was added, and the membranes gently agitated for 15 min at 55°C. The membranes were then blot dried taking care that they did not dry completely, wrapped in Gladwrap and autoradiographed (section 4.4.4).

4.6.7 Stripping probe off the membrane

In order to successfully remove probes, membranes were never permitted to dry during or after hybridisation and washing.

Membranes were stripped of the probe following the directions of the manufacturer (Amersham). Membranes were incubated at 45°C for 30 mins

in 0.4 M NaOH, in an agitating water bath. The membranes were transferred into a solution of 0.1 x SSC; 0.1% SDS; 0.2 M Tris-HCl (pH 7.5), and incubated for a further 15 min. Membranes were then blot dried on 3MM Whatman paper and heat sealed in plastic and stored at 4°C.

4.6.8 Interpretation of the probed membranes

Clones identified as positive for repeat DNA were picked from the master plates and grown O/N and mini-prepared (section 4.5.6). These clones were sequenced following the directions in section 4.4.

4.6.9 Design of microsatellite primers

Specific PCR primers for *Dugong dugon* complementary to the unique sequences flanking the microsatellites were designed following the recommendations for primer design in section 4.3.1. The sequences for these primers are listed in table 4.1.

4.6.10 PCR amplification of microsatellite loci

Microsatellites were amplified in five dugongs from the Torres Strait and five from Moreton Bay and run on an agarose check gel for products. Polymorphism in the size of these products was difficult to detect on an agarose gel. Consequently, the optimised PCRs were repeated in the presence of an end-labelled primer with the radioactive isotope [γP^{32}] - dATP ('hot PCR') and run on a denaturing acrylamide gel (section 4.4.3) which could detect differences in size by one base pair. The PCR conditions were optimised following the protocol outlined in section 4.3.3.

4.6.11 Hot PCR

A microsatellite primer (one of a pair) was 5' end-labelled as indicated in section 4.6.3 and a PCR carried out according to section 4.3.3. 4 µl of 'stop' (marker) dye (from a sequencing kit) was added at the end of the PCR run. 4 µl of each Hot PCR product was loaded onto a 6% denaturing acrylamide gel (see section 4.4.3) alongside 4 µl of a control reaction (from a sequencing kit), which acted as a size standard. After running for the required period of time (to enable visualisation of the PCR products in the expected size range), the gel was prepared (section 4.4.3) and an autoradiograph exposed (see section 4.4.4) giving a hard copy for interpretation.

4.7 READING AUTORADIOGRAPHS AND HANDLING OF SEQUENCE DATA WITH COMPUTER SOFTWARE

After developing the autoradiograph, the DNA sequences were entered, aligned and compared using the IBM computer package, ESEE (Version 1.04, Cabot & Beckenbach, 1989). ESEE was also used to translate the coding strand (of cytochrome *b*) into proteins. Sequences were also entered into GENBANK, a service available on the Internet. In addition, the Australian National Genomic Information Service (ANGIS, also available on the Internet) was used to search for common alignments. Being able to align conserved portions of the dugong sequence to other species ensured that I was investigating the correct region of the mitochondrial genome and allowed me to confirm that it was dugong. Computer software programs employed to investigate phylogenetic relationships and estimate measurements of gene flow are described in the relevant chapters.

CHAPTER FIVE

THE CYTOCHROME b GENE OF MITOCHONDRIAL DNA

In this chapter I detail the use of cytochrome *b* gene sequences to determine the following: 1) whether cytochrome *b* sequences would be suitable to examine intraspecific subdivision for the dugong, and 2) to estimate substitution rates and divergence times among sirenians.

5.1 INTRODUCTION

5.1.1 Function of the cytochrome b gene

The cytochrome *b* gene is probably the best known mitochondrial gene with respect to its structure and function. Understanding these traits is important to the interpretation of cytochrome *b* sequences for population and phylogenetic studies (Meyer, 1994).

Positioned 5' to the D-loop in mtDNA (figure 5.1), the cytochrome *b* gene encodes (see table 5.1 for the mammalian genetic code) one of the nine or ten electron carrier proteins that make up complex III of the mitochondrial oxidative phosphorylation system (Hatefi, 1985; Irwin *et al.*, 1991). Cytochrome *b* is the only cytochrome encoded in the mitochondrial genome that includes redox centres (quinone reaction sites Q_i and Q_o) involved in electron transfer (figure 5.2; Hatefi, 1985; Howell & Gilbert, 1988; Irwin *et al.*, 1991). The structure of cytochrome *b* (figure 5.2) was determined from mutational (di Rago *et al.*, 1990) and comparative studies (Howell & Gilbert, 1988; Howell, 1989).

The quinone reaction site Q_i is located on the proton input side of the mitochondrial membrane, and Q_o is located on the output side of the membrane (figure 5.2). Both Q_i and Q_o are highly conserved in the structural model of cytochrome *b* among mammals (Irwin *et al.*, 1991). The output side of the membrane is probably involved in redox catalysis (Ma *et al.*, 1993). The inner surface is composed mostly of the amino and carboxy

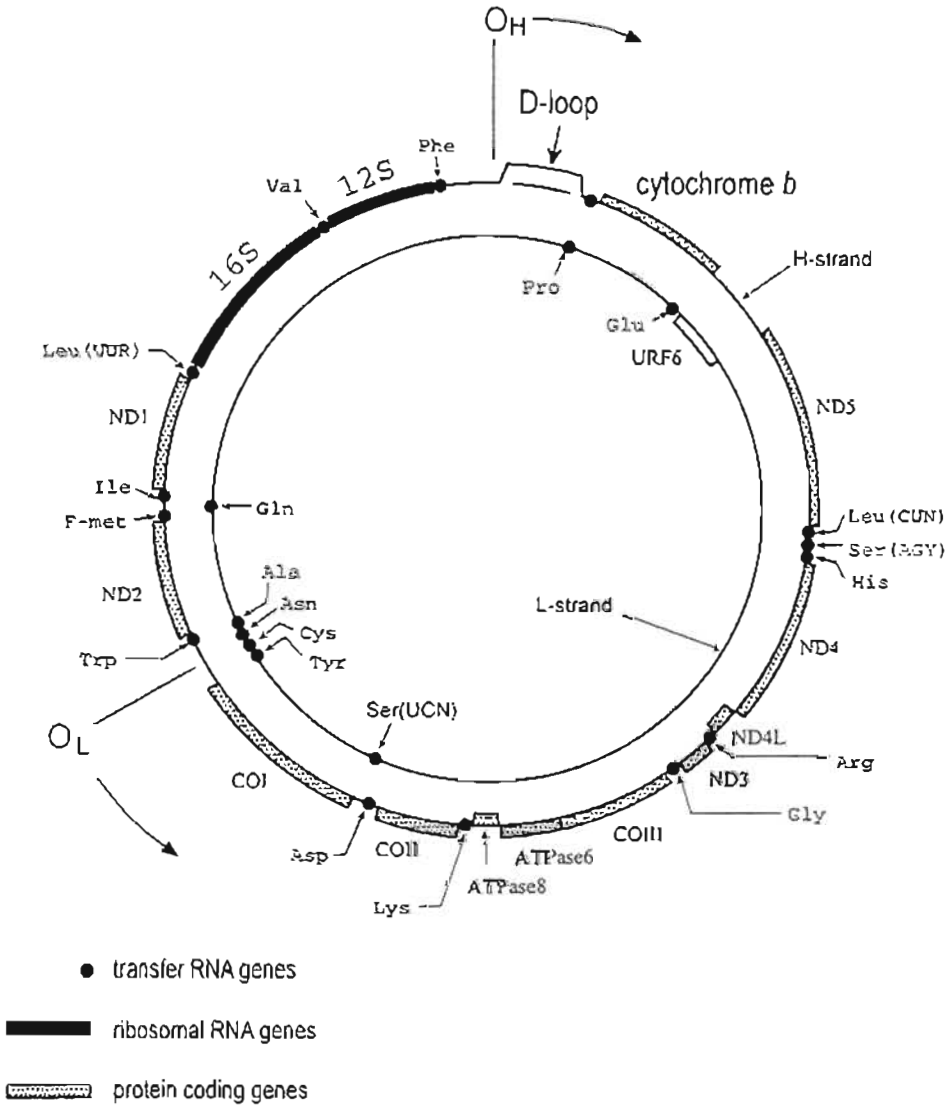


Figure 5.1: Map of the 16,000 base pair mammalian mitochondrial genome. The outer circle indicates those genes transcribed from the H (heavy) strand DNA and the inner circle indicates those genes transcribed from the L (light) strand DNA. O_H and O_L designate the origin of the H-strand and L-strand respectively. Whenever a space exists on one strand, it is filled by a gene on the other strand. In the designation of codons recognised by certain tRNAs, Y stands for pyrimidine, R stands for purine, and N stands for any base. ATPase 6 and 8 are components of the mitochondrial ATPase complex. COI, COII and COIII are cytochrome oxidase subunits. ND1-5 code for components of the respiratory chain NADH dehydrogenase. URF6 is an unidentified open reading frame (reproduced from Watson et al., 1987). Refer to table 5.1 for amino acid abbreviations.

Table 5.1: Genetic code of mammalian mitochondria. U stands for uracil, a similar base to thymine which is substituted when the DNA is read by RNA polymerase. The amino acids specified by the codons are abbreviated along with their single letter code (reproduced from Watson *et al.*, 1987).

Second Position

First Position (5' end)	U	C	A	G	Third Position (3' end)
U	UUU Phe F	UCU Ser S	UAU Tyr Y	UGU Cys C	U
	UUC Phe F	UCC Ser S	UAC Tyr Y	UGC Cys C	C
	UUA Leu L	UCA Ser S	UAA Stop *	UGA[α] Trp W	A
	UUG Leu L	UCG Ser S	UAG Stop *	UGG Trp W	G
C	CUU Leu L	CCU Pro P	CAU His H	CGU Arg R	U
	CUC Leu L	CCC Pro P	CAC His H	CGC Arg R	C
	CUA Leu L	CCA Pro P	CAA Gln Q	CGA Arg R	A
	CUG Leu L	CCG Pro P	CAG Gln Q	CGG Arg R	G
A	AUU Ile I	ACU Thr T	AAU Asn N	AGU Ser S	U
	AUC Ile I	ACC Thr T	AAC Asn N	AGC Ser S	C
	AUA[α] Met[β] M	ACA Thr T	AAA Lys K	AGA[α] Stop *	A
	AUG Met[β] M	ACG Thr T	AAG Lys K	AGG[α] Stop *	G
G	GUU Val V	GCU Ala A	GAU Asp D	GGU Gly G	U
	GUC Val V	GCC Ala A	GAC Asp D	GGC Gly G	C
	GUA Val V	GCA Ala A	GAA Glu E	GGA Gly G	A
	GUG Val V	GCG Ala A	GAG Glu E	GGG Gly G	G

[α] Codons that differ between the mitochondrial and universal genetic codes.

[β] Internal methionine is encoded by both AUG and AUA, initiating methionines are specified by AUG, AUA, AUU and AUC.

AMINO ACIDS

F	phenylalanine	T	threonine	D	aspartic acid
L	leucine	A	alanine	E	glutamic acid
I	isoleucine	Y	tyrosine	C	cysteine
M	methionine	H	histidine	W	tryptophan
V	valine	Q	glutamine	R	arginine
S	serine	N	asparagine	G	glycine
P	proline	K	lysine		

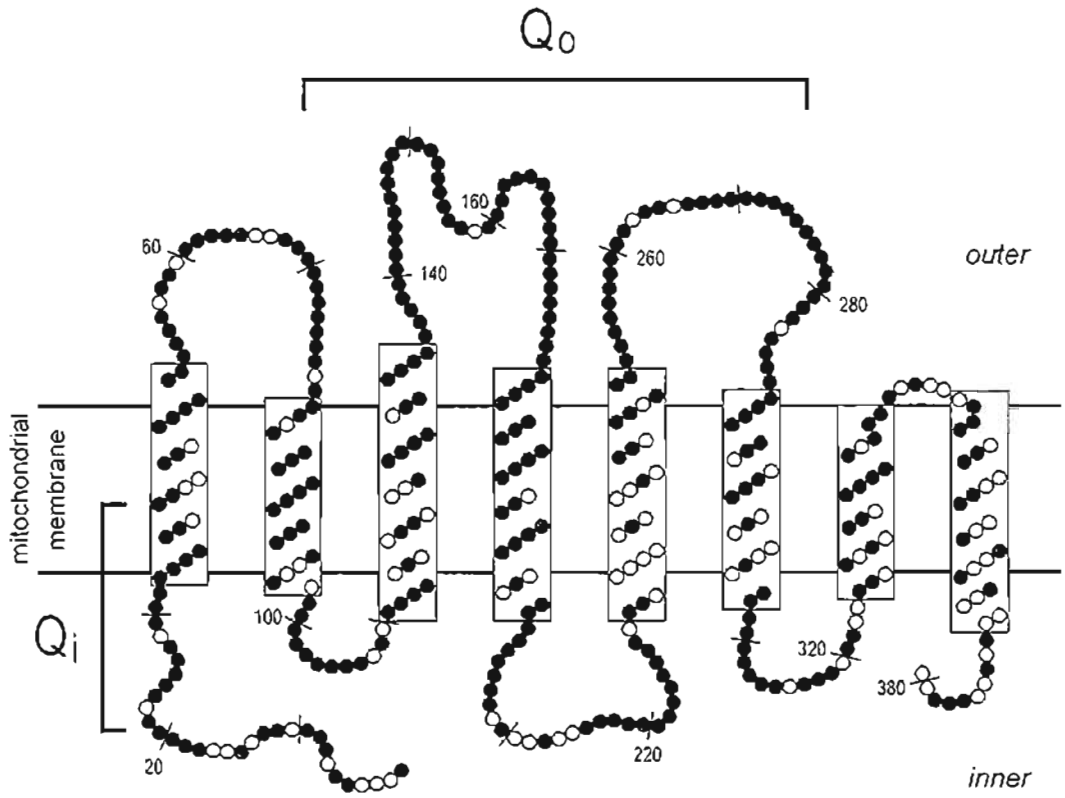
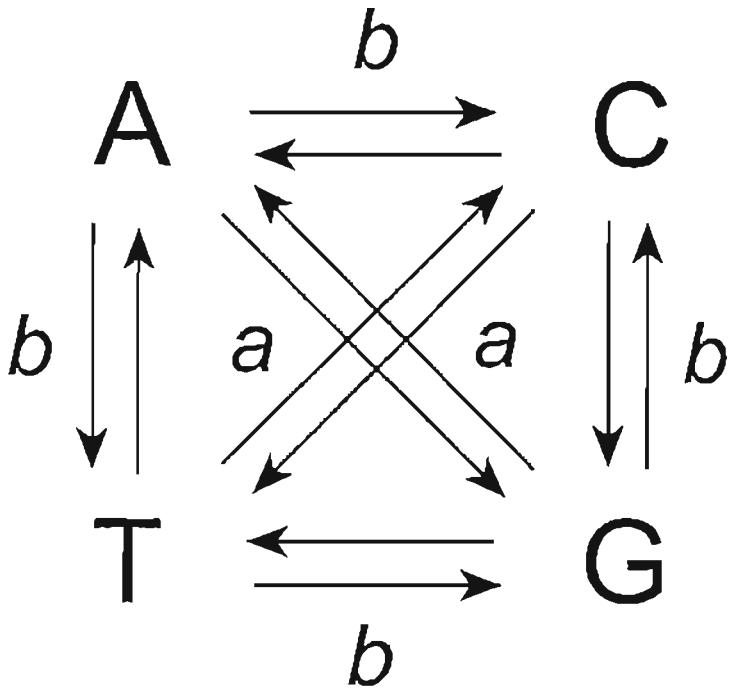


Figure 5.2: Structural model of mammalian cytochrome *b* (reproduced from Howell, 1989 and Irwin *et al.*, 1991). Each circle represents a single amino acid residue. Filled circles and open circles represent amino acids with high and low conservation between mammalian species, respectively. The segments of the protein that constitute the Qi and Qo redox centres are bracketed.



purine [A = adenine
G = guanine

C = cytosine
T = thymine] *pyrimidine*

Figure 5.3: **Nucleotide substitutions** (Nei, 1987).

a) transitions (A ⟷ G and T ⟷ C)

b) transversions (all others)

terminals of the protein and its functional importance is less defined (Irwin *et al.*, 1991; Ma *et al.*, 1993). Replacements observed in the transmembrane portion tend to be exchanges among hydrophobic residues (namely leucine (L), isoleucine (I) and valine (V)).

5.1.2 Substitution patterns in the cytochrome *b* gene

5.1.2.1 Uneven nucleotide frequencies

The occurrence of each nucleotide with an uneven frequency (bias) probably originates from selection or mutation pressure (Sueoka, 1988) and can be taxon-specific (Irwin *et al.*, 1991). Animal mitochondrial DNA is noted for an extreme bias in base composition at silent sites (Brown, 1985). Third positions have low content of the base guanine (G), second positions are rich in thymine (T), while the first position has an unbiased composition (Irwin *et al.*, 1991).

5.1.2.2 Transition: Transversion ratio

A general tendency for transitions to occur at a much higher frequency than transversions has been noted for both coding and non coding regions of DNA (Brown *et al.*, 1982; Cann & Wilson, 1983). Brown *et al.* (1982) attributed the predominance of transitions over transversions to the mutation process rather than to selection at the level of the gene products. The preference towards transitions at synonymous sites is most apparent for closely related groups (i.e., species within a genus) (Aquadro & Greenberg, 1983; Brown, 1985). Transversions become more apparent between more distant relatives (i.e., genera within a family, or order) (Thomas & Beckenbach, 1989). With increasing phylogenetic distance (i.e., between Orders or Classes of vertebrates), transition changes become saturated (i.e., the proportion of back mutations equals the number of forward mutations), and the ratio of transitions to transversions decreases until no difference in the ratio is detectable (Brown & Simpson, 1982). The transition (ts) to transversion (tv) ratio, quantified by pairwise comparisons among cytochrome *b* gene sequences between species, varies at each position within a codon (Irwin *et al.*, 1991). This mutation spectrum within the codon reflects differences in selection pressure (Irwin *et al.*, 1991). Irwin *et al.* (1991) estimated the average ts:tv ratio within mammalian mtDNA for silent positions was 10:1, or higher. In estimating divergence times among the Tethytherian clade (sirenians, proboscideans, hyracoideans), Ozawa *et al.*

(1997) assumed a ts:tv ratio of 10:1 when constructing the distance matrix from cytochrome *b* sequences.

5.1.2.3 Silent substitutions and replacement

Substitutions at the third position in a codon are usually silent and the most frequent. Silent substitutions (see figure 5.3 for the various types of substitution) are observed at a rate approximately 25 fold greater than at replacements (Irwin *et al.*, 1991). Therefore, estimates of an *overall* rate of change of the cytochrome *b* gene are of limited value (Irwin *et al.*, 1991). Silent transitions saturate within the first few million years (Myr). As a consequence, the observed increase in a difference between two sequences will be attributed to a tiny portion of the changing sites, which are the silent transversions and replacement substitutions (Irwin *et al.*, 1991). For this reason, when investigating phylogenetic relationships more than five Myr apart, the highly variable positions of a codon (i.e., first and especially the third) are often excluded (Irwin *et al.*, 1991; Meyer, 1994; for examples see Edwards *et al.*, 1991; Irwin & Arnason, 1994). However, as transversions at the third positions of a codon accumulate almost linearly with time, they can be used to estimate divergences greater than 5 Myrs assuming a rate of 0.5% per Myr (Miyamoto & Boyle, 1989; Irwin *et al.*, 1991). For divergences up to 25 Myrs (accounting transitions), a total rate of 10% per Myr can be estimated, using silent transversions at the third position of codons and assuming a ts:tv ratio of 10:1 (Brown *et al.*, 1982; Irwin *et al.*, 1991).

Substitutions at the first position in a codon are the next most frequent. Replacements at the first position usually result in amino acid changes that are less likely to cause functional impairment of the protein, than replacements at the second or third positions in a codon. Probably for this reason, replacements at the first position are observed at a higher frequency than at the second or third positions (Brown, 1985; Irwin *et al.*, 1991). Substitutions at the second position are the most conserved as they invariably lead to amino acid replacement. The rate of replacement at the first and second positions in a cytochrome *b* gene codon is estimated as approximately 0.4% per Myr (Irwin *et al.*, 1991).

5.1.3 The cytochrome *b* gene in within-species studies

Both replacements and silent transversions in the cytochrome *b* gene are useful for examining deep (i.e., greater than five Myr) phylogenetic relationships in mammals (Irwin *et al.*, 1991; Ma *et al.*, 1993; Irwin & Arnason, 1994), birds (Edwards *et al.*, 1991) and other classes of vertebrates (Kocher *et al.*, 1989; Meyer & Wilson, 1990). Cytochrome *b* is also useful at shallower phylogenetic levels (less than five Myr). Using 402 base pairs (bp) of the cytochrome *b*, and all positions of a codon corrected by maximum-likelihood analysis (for substitution rate variability), Geffen *et al.* (1992) reported an average sequence divergence of 14.3% among ten species of fox-like canids and estimated divergences among species to be up to about five Myr. The power of cytochrome *b* data can be increased by the inclusion of other genetic information. For example, the control region was used in conjunction with the cytochrome *b* gene to resolve the phylogenetic relationship of north American ursids (*Ursus maritimus*, *U. arctos* and *U. americanus*) which were estimated to have diverged around four Myr ago (Shields & Kocher, 1991).

Interspecific pairwise comparisons of cytochrome *b* gene sequences enable estimates of divergence times to be obtained by assuming a substitution rate. Alternatively, substitution rates can be estimated assuming a divergence time (e.g., from fossil data). Ozawa *et al.* (1997) used this approach to estimate divergence times among the Tethytheria (i.e., sirenians, proboscideans, and hyracoideans). To calculate substitution rates and divergence times for sirenians, much time and expense in the laboratory would be saved if only portion of a gene needed to be sequenced. Edwards *et al.* (1991) found that amplifying 924 bp of the cytochrome *b* genes of birds, compared to 307 bp therein, more than trebled the 'phylogenetic resolving power'. I wanted to confirm whether examining just a portion (i.e., around 250 bases) of the cytochrome *b* gene is adequate to resolve the major population-level partitions in dugongs. To achieve this, I reexamine estimates of substitution rate and divergence times by comparing 225 bases of West Indian manatee cytochrome *b* sequence (Bradley *et al.*, 1993), to 1005 bases of the West Indian manatee cytochrome *b* sequenced by Ozawa *et al.* (1997), and the entire cytochrome *b* of the dugong (Irwin & Arnason, 1994). That is, both a short sequence (225 bases) and longer sequence (1005 bases) were compared between the West Indian manatee and dugong.

5.1.4 The cytochrome *b* gene in intraspecific studies

In some species, the cytochrome *b* gene has the level of variation suitable for an intraspecific study. In general, expected within-species variation is usually less than 5% nucleotide divergence, whereas interspecific variation is expected to be greater than 5% (Irwin *et al.*, 1991). Reported cases of large intraspecific variation include South American rodents (0.25 to 8% intraspecific variation in the cytochrome *b* sequence (Smith & Patton, 1991)), and the eastern African black-backed jackal (8%, (Wayne *et al.*, 1990)). Randi *et al.* (1994) used part of the cytochrome *b* sequence in conjunction with a portion of the control region to examine genetic structure of the European brown bear (*Ursus arctos*), which was monomorphic within a population and had an average among-population divergence of 2.8% for cytochrome *b* haplotypes. However, they were polymorphic for D-loop haplotypes with 10.7% average nucleotide divergence (Randi *et al.*, 1994).

Lento *et al.* (1994) used cytochrome *b* haplotypes to distinguish between the Western Australian and the New Zealand populations of New Zealand fur seals (*Arctocephalus forsteri*). They were, however, unable to distinguish between the east and west New Zealand populations using cytochrome *b* haplotypes, and suggested further investigation into the population structure using (mtDNA) control region sequences. With the exception of one individual which had a sequence divergence range of 3.2% to 4%, the haplotypic variation of *A. forsteri* among Western Australian and New Zealand populations (0.3% to 0.8%), is within the expected range of intraspecific variation for the cytochrome *b* gene.

As the cytochrome *b* gene is known to contain sufficient sequence variation for within-specific studies of some species, it was investigated primarily for its suitability to recognise intraspecific units for the dugong. This was achieved by examining the degree of variation among cytochrome *b* sequences of three dugongs from populations separated by substantial geographic distance (i.e., Exmouth Bay and Torres Strait (this study), and Moreton Bay (Irwin & Arnason, 1994), see figure 3.2 for localities).

5.1.5 Cytochrome *b* in sirenians

There are two objectives to this chapter:

1) To examine whether cytochrome *b* sequences of the dugong are sufficiently variable for recognition of intraspecific subdivision.
2) To determine whether a portion of the cytochrome *b* gene was as informative as a longer portion of the gene. Sequencing the entire gene of many individuals for a population study would be tedious, expensive and labour intensive. Therefore, I wanted to assess whether a portion of the cytochrome *b* gene (225 bp) was as informative as the (near) entire (1005 bp) gene in resolving major population-level partitions. The total length of the cytochrome *b* gene is 1140 bp. Complete or nearly complete cytochrome *b* sequences are now available for the dugong, West Indian manatee, and Steller's sea cow (Bradley *et al.*, 1993; Irwin & Arnason, 1994; Ozawa *et al.*, 1997).

5.2 METHODS

5.2.1 Sequencing the cytochrome *b* gene of mtDNA

DNA was extracted and purified from one dugong from the Torres Strait (sample T677, table 7.2) and one dugong from Exmouth Bay, Western Australia (sample WA, table 7.2) using techniques outlined in sections 4.1 and 4.2. Portions of the cytochrome *b* gene were amplified (section 4.3), cloned (section 4.5) and sequenced (section 4.4) initially using primers designed by Kocher *et al.* (1989). Then I designed (section 4.3.1) dugong-specific primers (table 4.1) for segments of the cytochrome *b* sequence not amplified by the primers of Kocher *et al.* (1989). One clone from each individual was sequenced and aligned (section 4.7) to the complete cytochrome *b* gene of a dugong from Moreton Bay (Irwin & Arnason, 1994), as well as 1005 bp of the cytochrome *b* gene of Steller's sea cow (Ozawa *et al.*, 1997), and 225 bp & 1005 bp of the West Indian manatee (Bradley *et al.* (1993) and Ozawa *et al.* (1997) respectively).

A PCR product mix typically contains mutations from *Taq* polymerase error (Pääbo & Wilson, 1988; Saiki *et al.*, 1988). With the exception of occasions when the template DNA is represented by only a few copies, direct

sequencing a PCR product is unlikely to detect *Taq* polymerase error, as any misincorporated nucleotides are concealed by the multiple correct copies that are coamplified. A cloned PCR product on the other hand, will reveal misincorporations more readily, as any errors present in the clone are amplified by the bacterial host and subsequently sequenced (Pääbo & Wilson, 1988; Saiki *et al.*, 1988). To recognise misincorporation error in clones, it is recommended that three clones be sequenced and a consensus sequence (which has a greater likelihood of being the actual sequence) be used (Irwin *et al.*, 1991). The small percentage of base variation expected from 'cloning artefacts' was not of sufficient concern in assessing whether the cytochrome *b* would be sufficiently variable for an intraspecific level study, and I did not sequence three clones for this reason.

5.2.2 Substitution rates and time divergences

The Kimura two-parameter algorithm (Kimura, 1980) accommodates different frequencies in transitions and transversions. This model was used to produce measures of genetic distance from pairwise comparisons with a ts:tv ratio of 10:1 specified, using the program DNADIST in PHYLIP (Macintosh, Version 3.5c, Felsenstein, 1989). The ts:tv ratio of 10:1 is assumed as observed ts:tv ratios rarely approximate this value because of saturation at sites.

Substitution rates (substitutions/site/Myr) were estimated from the measures of distance among the dugong (this study; Irwin & Arnason, 1994) and West Indian manatee (Bradley *et al.*, 1993; Ozawa *et al.*, 1997) cytochrome *b* sequences (substitutions/site), assuming various divergence times (Myr) proposed from immunological (Rainey *et al.*, 1984) and fossil data (Domning, 1994).

Conversely, time divergences between the dugong and West Indian manatee were estimated from the distance measures assuming the following substitution rates: 1) 0.11% per Myr at the first and second position assuming a ts:tv ratio of 10:1 (Ozawa *et al.*, 1997), 2) 0.4% per Myr for replacement substitutions at the first and second positions of a codon (Irwin *et al.*, 1991), 3) 0.5% per Myr for silent transversions at the third position of a codon (Irwin *et al.*, 1991), 4) 10% per Myr for total rate of divergence at the third position of a codon assuming a ts:tv ratio of 10:1 (Irwin *et al.*, 1991), and 5) the rate shown to be the most consistent between

comparisons made with the shorter (225 bp) and longer (1005 bp) segments of cytochrome *b* sequences.

These calculations were done for both the near entire gene and for a segment comprising 225 bp. The shorter segment evaluated corresponds to the tract from the West Indian manatee sequenced by Bradley *et al.* (1993).

5.3 RESULTS

5.3.1 Comparison between dugong sequences

The total of 1085 bases (composite of the cytochrome *b* gene) sequenced from a Torres Strait and an Exmouth Bay (Western Australian) dugong were aligned with the complete (1140 bases) cytochrome *b* gene for the dugong sequenced by Irwin & Arnason (1994), 225 and 1005 bases of the West Indian manatee (Bradley *et al.*, 1993 and Ozawa *et al.*, 1997 respectively), and 1005 bases of the Steller's sea cow (Ozawa *et al.*, 1997).

The sections of cytochrome *b* gene I sequenced for the Torres Strait and the Exmouth Bay dugongs do not overlap (figure 5.4). However, both sections were found to be identical to Irwin & Arnason's (1994) dugong sequence with the exception of three sites: 225, 386 and 1022 in figure 5.4 (corresponding with codons 75, 129 and 341 in figure 5.4 respectively). The variable base at position 225 is a silent CT transition in the third position of the conserved codon #75 (figures 5.2 & 5.4). The CT transition at site 386 is the second base position of codon #129 (figure 5.4) resulting in a replacement from methionine (M) to threonine (T). The third variation at site 1022 in codon #341 (figure 5.4) is a thymine (T) insertion. Assuming that the three differences among the dugong sequences are due to *Taq* error, an estimated misincorporation rate of 2.6×10^{-4} bases per cycle was obtained using the following formula (Saiki *et al.*, 1988):

$$M = 2\left(\frac{f}{d}\right)$$

Where M equals the misincorporation rate, f is the observed error frequency (i.e., number of observed errors divided by the number of bases sequenced) and d is the number of duplications (i.e., 30 PCR cycles).

```
                    ATG = CYT B GENE START >
trl. 1) D.dugon      M   T   N   I   R   K   S   H   P   L   I   K   I   L   N   15
trl. H.gigas         .   .   .   .   .   .   T   .   .   .   .   .   .   .   .   15
trl. 2) T.manatus    .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   15
1) Dugong dugon      ATG ACC AAC ATC CGA AAA TCA CAC CCA CTA ATC AAA ATC CTA AAC  45
Hydrodamalis gigas   ... ... ... ..T ... ... A.. ..T ... ..G ... ... ... ..G ..T  45
2) T.manatus         ... ... ... ..T ... ... ... ... ... ... ... ... ... T.G ..T  45

trl. 1) D.dugon      N   S   F   I   D   L   P   T   P   V   N   I   S   S   W   30
trl. H.gigas         D   .   .   .   .   .   .   .   .   T   .   .   .   .   .   30
trl. 2) T.manatus    D   .   .   .   .   .   .   .   .   T   .   .   .   .   .   30
1) Dugong dugon      AAC TCC TTC ATT GAC CTC CCT ACC CCC GTA AAT ATC TCA TCA TGA  90
2) Dugong dugon                                                  .. ...          5
Hydrodamalis gigas   G.. ..A ... ..C ... ... ..C ..T ..A AC. ..C ... ... ... ...  90
2) T.manatus         G.. ... ... ..C ... T.G ..A ..T ..A AC. ..C ... ... ... ...  90

trl. 1) D.dugong     W   N   F   G   S   L   L   G   A   C   L   I   I   Q   I    45
trl. H.gigas         .   .   .   .   .   .   .   .   .   .   .   .   .   .   .    45
trl. 2) T.manatus    .   .   .   .   .   .   .   .   .   .   .   .   .   .   .    45
1) Dugong dugon      TGA AAC TTT GGC TCC CTA CTC GGG GCA TGC CTG ATT ATT CAA ATT  135
2) Dugong dugon      ... ... ... ... . . ... ... ... ... ... ... ... ... ... ...   50
Hydrodamalis gigas   ..G ... ... ..T ... T.. ..T ..A ..G ... ..A ... ... ... ...  135
2) T.manatus         ... ... ... ... ..A ... ..T ... ..G ... ..A ..C ... ... ...  135
```

Figure 5.4: The cytochrome *b* gene sequence of sirenian mtDNA. The sequence is split into divisions of three corresponding to a single codon. The translated sequence is shown in the single letter code (see table 5.1 for the genetic code). A period denotes a common nucleotide. A hyphen denotes the absence of a base at that position. Differences from the reference sequence are indicated by the variant base (see figure 5.3 for the types of substitution). Numbers at the end of the translated sequence and the DNA sequence, indicate the codon and site (base) position respectively for that sequence. The following details the source of each sequence.

`trl. dugong` Translation of the dugong sequence reported by Irwin & Arnason (1994).

`trl. H.gigas` Translation of the Steller's sea cow sequence reported by (Ozawa *et al.*, 1997).

`trl. 2) T.manatus` Translation of the West Indian manatee sequence reported by (Ozawa *et al.*, 1997).

`1) Dugong dugon` A Moreton Bay dugong sequence provided by Arnason (personal communication) and Irwin & Arnason (1994). It is also the reference sequence for this alignment. Genbank accession number: DDU07564.

`2) Dugong dugon` Exmouth Bay dugong (sample WA). This study.

`3) Dugong dugon` Torres Strait dugong (sample T677). This study.

`Hydrodamalis gigas` Steller's sea cow (Ozawa *et al.*, 1997). Genbank accession number: D83049.

`1) T.manatus` West Indian manatee (Bradley *et al.*, 1993).

`2) T.manatus` West Indian manatee (Ozawa *et al.*, 1997). Genbank accession number: D85050.

This figure continues for an additional 3 pages.

```
trl. D.dugon                L  T  G  L  F  L  A  I  H  Y  T  S  D  T  L   60
trl. H.gigas                .  .  .  .  .  .  .  .  .  .  .  .  .  .  S   60
trl. 2) T.manatus           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .   60
1) Dugong dugon             CTC ACA GGA TTA TTC CTG GCC ATA CAC TAC ACA TCA GAC ACA CTA  180
2) Dugong dugon             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...   95
Hydrodamalis gigas          ... ... ... C . ... T.. ... ... ... ..T ..G ..C ..T ..G TC.  180
1) T.manatus                ... ... ... ... ... ..A ... ... ... ... ... ... ... ..G ...   42
2) T.manatus                ... ... ... ... ... ..A ... ... ... ... ... ... ... ..G ...  180

                                                                        site 225
trl. dugong                 T  A  F  S  S  V  T  H  I  C  R  D  V  N  Y   75
trl. H.gigas                .  .  .  .  .  .  A  .  .  .  .  .  .  .  .   75
trl. 2) T.manatus           .  .  .  .  .  .  A  .  .  .  .  .  .  .  .   75
1) Dugong dugon             ACC GCA TTC TCC TCA GTA ACC CAT ATT TGC CGG GAT GTA AAC TAC  225
2) Dugong dugon             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T  140
Hydrodamalis gigas          ..T ... ..T ... ... ..G G.. ... ..C ..T ..A ... ... ... ...  225
1) T.manatus                ... ... ... ..T ... ... G.. ..C ... ... ..A ..C ..C ... ..T   87
2) T.manatus                ... ... ... ..T ... ... G.. ..C ... ... ..A ..C ..C ... ..T  225

                                                                    site 270
trl. 1) D.dugon             G  W  I  I  R  Y  L  H  A  N  G  A  S  I  F   90
trl. H.gigas                .  .  .  .  .  .  .  .  .  .  .  .  .  .  .   90
trl. 2) T.manatus           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .   90
1) Dugong dugon             GGC TGA ATT ATT CGA TAT CTT CAC GCT AAC GGG GCA TCA ATA TTC  270
2) Dugong dugon             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  185
Hydrodamalis gigas          ... ... ... ..C ... ... ... ... ..C ... ..A ... ... ... ..T  270
1) T.manatus                ..T ... ..C ... ..C ..C ..C ..T ..C ... ..A ... ... ... ...  132
2) T.manatus                ..T ... ..C ... ..C ..C ..C ..T ..C ... ..A ... ... ... ..T  270

trl. 1) D.dugon             F  L  C  L  Y  A  H  I  G  R  G  I  Y  Y  G  105
trl. H.gigas                .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  105
trl. 2) T.manatus           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  105
1) Dugong dugon             TTC CTA TGC CTC TAC GCC CAC ATT GGA CGC GGA ATC TAT TAC GGC  315
2) Dugong dugon             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  230
Hydrodamalis gigas          ... T.G ... ... ... ..T ... ..C ... ... ... ..T ..C ... ...  315
1) T.manatus                ... ... ..T ... ... ... ... ... ... ..T ... ... ... ... ...  177
2) T.manatus                ... ... ..T ... ... ... ... ... ... ..T ... ... ... ... ...  315

trl. 1) D.dugon             S  Y  L  Y  P  E  T  W  N  I  G  I  V  L  L  120
trl. H.gigas                .  .  .  .  .  .  .  .  .  .  .  .  I  .  .  120
trl. 2) T.manatus           .  .  .  .  S  .  .  .  .  .  .  .  I  .  .  120
1) Dugong dugon             TCA TAC CTA TAT CCA GAA ACC TGA AAC ATT GGT ATC GTA CTG CTA  360
2) Dugong dugon             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  275
Hydrodamalis gigas          ... ..T ... ..C ... ..G ... ... ... ... ..C ... A.. ..A ...  360
1) T.manatus                ..C ... T.. ... T.. ... ... ... ... ... ..A ... A.C ..A ...  222
2) T.manatus                ..C ... T.. ... T.. ... ... ... ... ... ..A ... A.C ..A ...  360

                                                    site 386
trl. 1) D.dugon             L  T  V  I  A  T  A  F  I  G  Y  V  L  P  W  135
trl. H.gigas                .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  135
trl. 2) T.manatus           .  .  I  .  .  .  .  .  .  .  .  .  .  .  .  135
1) Dugong dugon             CTC ACA GTT ATA GCT ACT GCC TTC ATA GGG TAC GTC CTC CCA TGA  405
2) Dugong dugon             ... ... ... ...                                            287
3) Dugong dugon                         ... ... ... ... .C. ... ... ... ... ... ...   33
Hydrodamalis gigas          ... ... ..G ... ..C ... ..T ... ... ..A ... ... ... ..G ...  405
1) T.manatus                ...                                                        225
2) T.manatus                ... ... A.. ... ..C ..C ..T ... ... ..A ... ... ... ... ..G  405
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Figure 5.4: Continued.

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trl. 1) D.dugon          G   Q   I   S   F   W   G   A   T   V   I   T   N   L   L   150
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   150
trl. 2) T.manatus        .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   150
1) Dugong dugon          GGA CAA ATA TCA TTC TGA GGA GCA ACC GTT ATT ACT AAC CTC CTG  450
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...   78
Hydrodamalis gigas       ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ..T ...  450
2) T.manatus             ... ..G ... ... ... ... ..G ... ..T ..A ..C ..C ... ..T ..A  450

trl. 1) D.dugon          S   A   I   P   Y   I   G   T   N   L   V   E   W   V   W   165
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   165
trl. 2) T.manatus        .   .   .   .   .   .   .   .   D   .   .   .   .   I   .   165
1) Dugong dugon          TCA GCT ATC CCC TAC ATC GGC ACC AAC CTA GTC GAA TGA GTT TGA  495
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  123
Hydrodamalis gigas       ..T ... ..T ... ..T ... ... ... ..T ..G ..A ..G ... ..C ...  495
2) T.manatus             ... ..C ... ... ... ... ... ... G.. ... ..A ... ... A.C ...  495

trl. 1) D.dugon          G   G   F   S   V   D   K   A   T   L   T   R   F   F   A   180
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   180
trl. 2) T.manatus        .   .   .   .   .   .   .   .   .   .   .   .   .   .   V   180
1) Dugong dugon          GGG GGA TTC TCA GTA GAC AAA GCC ACC CTC ACC CGA TTC TTC GCC  540
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  168
Hydrodamalis gigas       ..T ... ... ..G ..G ... ... ... ... ..T ... ... ... ... ..T  540
2) T.manatus             ..T ... ... ... ... ... ... ..T ... ... ... ... ..T ... .TG  540

trl. 1) D.dugon          L   H   F   I   L   P   F   I   V   T   A   L   V   I   V   195
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   A   .   195
trl. 2) T.manatus        .   .   .   T   .   .   .   V   .   .   .   .   .   A   .   195
1) Dugong dugon          CTA CAC TTC ATC CTA CCC TTC ATC GTA ACC GCC CTA GTA ATA GTC  585
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  213
Hydrodamalis gigas       T.G ... ... ... ... ..A ... ... ... ... ... ... ... GC. ..T  585
2) T.manatus             T.. ... ... .C. ... ..T .. G.. ... ... ... ... ... GCC ...  585

trl. 1) D.dugon          H   L   L   F   L   H   E   T   G   S   N   N   P   T   G   210
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   210
trl. 2) T.manatus        .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   210
1) Dugong dugon          CAC TTA CTA TTC CTC CAC GAA ACA GGC TCC AAC AAC CCC ACG GGA  630
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  258
Hydrodamalis gigas       ..T C.. T.. ... ..T ... ... ... ..A ... ... ..T ..T ..A ..C  630
2) T.manatus             ..T C.C ... ... ..T ... ..G ... ..G ... ... ... ... ..A ..C  630

trl. 1) D.dugon          L   I   S   D   S   D   K   I   P   F   H   P   Y   Y   S   225
trl. H.gigas             .   V   .   N   .   .   .   .   .   .   .   .   .   .   .   225
trl. 2) T.manatus        .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   225
1) Dugong dugon          CTG ATC TCC GAC TCA GAC AAA ATC CCA TTC CAC CCA TAT TAT TCA  675
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  303
Hydrodamalis gigas       T.A G.. ... A.. ... ... ..G ... ... ... ... ... ..C ..C ..T  675
2) T.manatus             ..A ... ... ..T ..C ... ... ..T ... ..T ..T ... ..C ..C ...  675

trl. 1) D.dugon          V   K   D   L   L   G   L   F   L   L   I   L   V   L   L   240
trl. H.gigas             T   .   .   F   .   .   .   .   .   .   .   .   .   .   .   240
trl. 2) T.manatus        I   .   .   .   .   .   .   .   .   .   .   .   I   .   .   240
1) Dugong dugon          GTC AAA GAC CTC CTA GGC CTA TTC CTC CTC ATT CTA GTC TTA CTC  720
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  348
Hydrodamalis gigas       AC. ... ..T T.. ... ... ... ..T ... ... ..C ... ... C.T ...  720
2) T.manatus             A.. ... ... ... ... ... ... ..T ... ... ..C ... A.. C.. ...  720

trl. 1) D.dugon          L   L   T   L   F   S   P   D   I   L   G   D   P   D   N   255
trl. H.gigas             .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   255
trl. 2) T.manatus        .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   255
1) Dugong dugon          CTA CTA ACC CTG TTC TCC CCG GAC ATA CTG GGA GAC CCA GAC AAC  765
3) Dugong dugon          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...  393
Hydrodamalis gigas       ..C ..G ... ..A ... ... ... ..T ..G ..A ... ... ..G ... ..T  765
2) T.manatus             ... ..G ... T.A ... ... ..A ... ..G T.A ... ... ... ... ...  765
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Figure 5.4: Continued.

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trl. 1) D.dugon      Y  T  P  A  N  P  L  N  T  P  P  H  I  K  P  270
trl. H.gigas         .  .  .  .  .  .  .  S  .  .  .  .  .  .  .  270
trl. 2) T.manatus    .  .  .  .  .  .  .  S  .  .  .  .  .  .  .  270
1) Dugong dugon      TAC ACA CCA GCC AAC CCA CTA AAC ACC CCT CCC CAC ATT AAA CCA  810
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  438
Hydrodamalis gigas   ... ... ..T ... ... ... ... .G. ... ..A ... ... ..C ...  810
2) T.manatus         ... ... ..C ..A ..T ... ... .G. ... ..A ... ... ... ..G  810

trl. 1) D.dugon      E  W  Y  F  L  F  R  Y  A  I  L  R  S  I  P  285
trl. H.gigas         .  .  .  .  .  .  A  .  .  .  .  .  .  .  .  285
trl. 2) T.manatus    .  .  .  .  .  .  A  .  .  .  .  .  .  .  .  285
1) Dugong dugon      GAA TGA TAC TTT CTA TTC CGA TAC GCT ATC CTC CGA TCT ATC CCT  855
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  483
Hydrodamalis gigas   ... ..G ... ..C ... ... GCG ... ... ... ..T ... ..C ... ..C  855
2) T.manatus         ... ..G ... ..C ... ..T GC. ... ..C ... ..T ... ..C ... ...  855

trl. 1) D.dugon      N  K  L  G  G  V  L  A  L  V  L  S  I  L  I  300
trl. H.gigas         .  .  .  .  .  .  .  .  .  I  .  .  .  .  .  300
trl. 2) T.manatus    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  300
1) Dugong dugon      AAT AAA CTA GGC GGC GTG TTA GCC CTC GTA CTC TCC ATC CTA ATC  900
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  528
Hydrodamalis gigas   ..C ... T.. ..T ..T ..A C.. ... ..T A.C ..A ... ... T.. ...  900
2) T.manatus         ..C ... ... ..T ..G ..C ... ... ..A ..T ... ..T ... ... ..T  900

trl. 1) D.dugon      L  A  L  L  P  L  L  H  T  S  K  Q  R  S  L  315
trl. H.gigas         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  315
trl. 2) T.manatus    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  315
1) Dugong dugon      CTA GCG CTC CTC CCA CTC CTC CAC ACA TCC AAA CAA CGA AGC CTA  945
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  573
Hydrodamalis gigas   ... ... ... ..T ... ..T ... ..T ... ... ... ... ... ..G  945
2) T.manatus         ... ..C ... ... ..T ... ... ... ... ... ... ..C ... ...  945

trl. 1) D.dugon      S  F  R  P  L  S  Q  C  L  F  W  I  L  V  A  330
trl. H.gigas         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  330
trl. 2) T.manatus    L  .  .  .  .  .  .  .  .  .  .  .  .  .  T  330
1) Dugong dugon      TCA TTC CGA CCT CTA AGC CAA TGC CTC TTT TGA ATT CTG GTA GCC  990
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  618
Hydrodamalis gigas   ... ..T ..C ..C ..C ... ... ... ... ..C ... ... ..A ... ...  990
2) T.manatus         .TG ... ..G ..C ... ... ... ... ..A ..C ... ..C ..A ... A..  990

                                                    site 1022
trl. 1) D.dugon      D  L  I  T  L  T  W  I  G  G  (Q)  P  V  E  H  345
trl. H.gigas         .  .  .  .  .  .                              335
trl. 2) T.manatus    .  .  .  .  .  .                              335
1) Dugong dugon      GAC CTG ATC ACA CTC ACA TGA ATC GGC GGC C-AA CCA GTA GAA CAT 1035
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... .T.. ... ... ... ...  663
Hydrodamalis gigas   ... ..A ..T ... ..T                           1005
2) T.manatus         ... ..A ... ... ..T                           1005

trl. 1) D.dugon      P  Y  I  I  I  G  Q  L  A  S  I  L  Y  F  S  360
1) Dugong dugon      CCC TAC ATC ATC ATC GGC CAA CTA GCC TCA ATC CTG TAC TTC TCC 1080
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  708

trl. 1) D.dugon      I  I  L  I  F  M  P  I  A  G  L  I  E  N  H  375
1) Dugong dugon      ATC ATC CTC ATC TTT ATG CCC ATC GCA GGC CTA ATT GAA AAT CAC 1125
3) Dugong dugon      ... ... ... ... ... ... ... ... ... ... ... ... ... ...  753

                  STOP CODON = AGG
trl. 1) D.dugong     L  L  K  W  *                                 380
1) Dugong dugon      CTA CTT AAA TGA AGG                           1140
3) Dugong dugon      ... ... ... ... ...                           798
```

Figure 5.4: Continued.

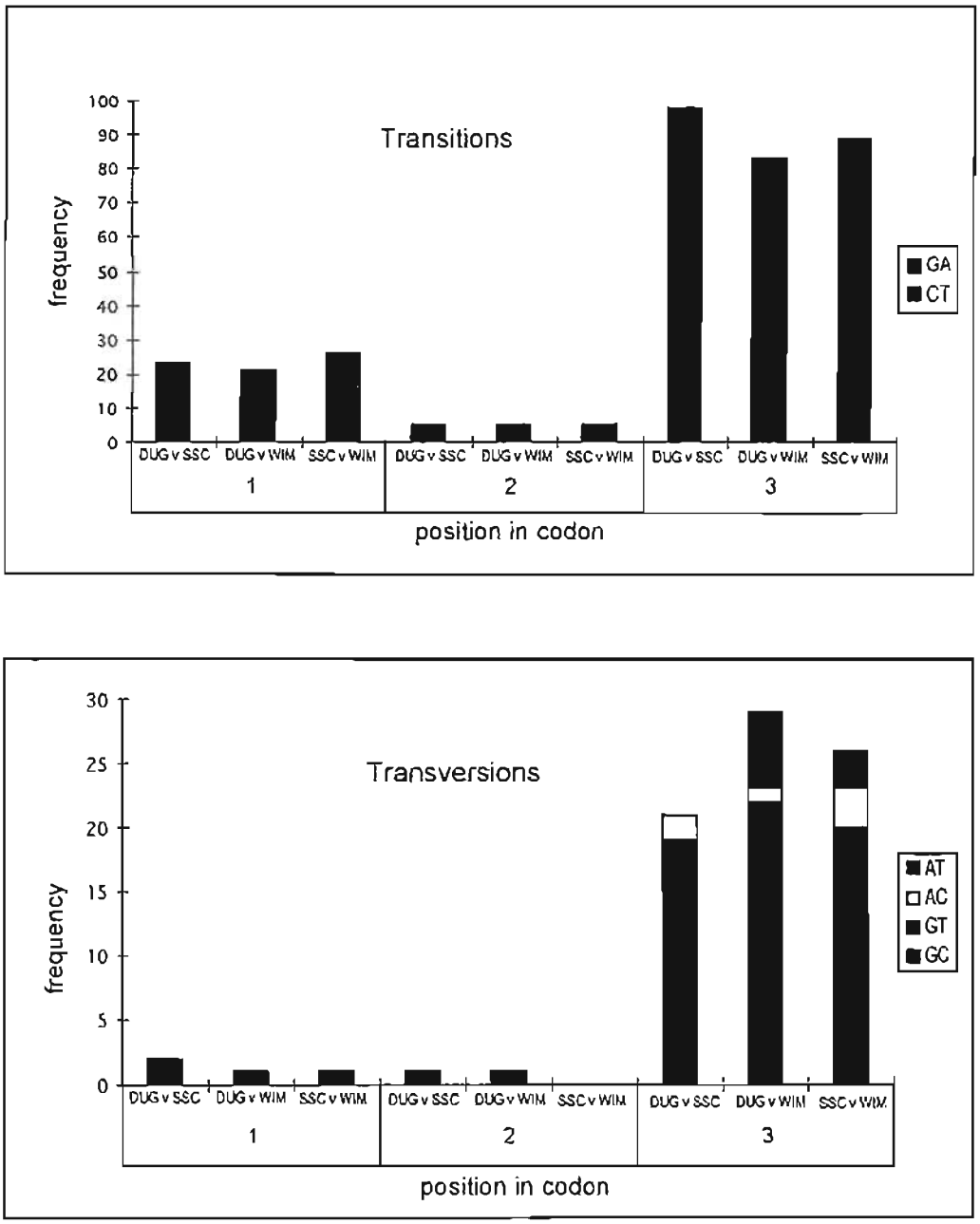

Figure 5.5: Frequency of substitutions observed at each position in a codon among sirenian cytochrome *b* sequences. The proportions of transitions and transversions among the dugong (DUG), West Indian manatee (WIM) and Steller's sea cow (SSC) by comparing the 1005 bp of cytochrome *b* sequences published by Irwin & Arnason (1994) and Ozawa *et al.* (1997). Substitutions were greatest at the third position of a codon >> first > second.

5.3.2 Comparison between the dugong, Steller's sea cow and West Indian manatee

No size variation exists in the 1005 bases of the cytochrome *b* gene compared between the dugong (Irwin & Arnason, 1994), Steller's sea cow and West Indian manatee (Bradley *et al.*, 1993; Ozawa *et al.*, 1997). Both published sequences for the West Indian manatee cytochrome *b* (Bradley *et al.*, 1993; Ozawa *et al.*, 1997) were identical in the 225 bases of sequence aligned, with the exception of one transition at site 270 (figure 5.4).

A total of 150 nucleotide differences were observed between the dugong and Steller's sea cow, and 142 differences between the dugong and West Indian manatee (figure 5.4; Ozawa *et al.*, 1997). The frequency of transitions was greatest at the: third >> first > second position in a codon (table 5.2 & figure 5.5). Transversions were greatest at the third position in a codon (table 5.2 & figure 5.5). The ts:tv ratios at the third position among the sirenian sequences (table 5.2) are lower than the predicted ts:tv ratio of 10:1 (Irwin *et al.*, 1991) suggesting the saturation of sites.

Table 5.2: Comparison of sequence statistics among 1005 bp of sirenian cytochrome *b* genes. The frequency of substitutions observed at each position in a codon from pairwise comparisons of the dugong (Irwin & Arnason, 1994), Steller's sea cow, and West Indian manatee (Ozawa *et al.*, 1997). No replacements (R) were observed for third position substitutions. Any substitution observed at the second position was a replacement.

Position in codon	*Dugong vs Steller's sea cow*			*Dugong vs West Indian manatee*			*West Indian manatee vs Steller's sea cow*		
	ts	**tv**	**R**	**ts**	**tv**	**R**	**ts**	**tv**	**R**
1st	23	2	12	21	1	13	26	1	13
2nd	6	1	7	6	1	7	5	0	5
3rd	98	21	0	83	29	0	89	32	0
total	**127**	**24**	**16[a]**	**110**	**31**	**19[a]**	**120**	**33**	**17[a]**
ts:tv ratio		5:1			4:1			4:1	

[a]On occasion, more than one substitution contributed to a replacement.

From pairwise comparisons, 16 replacements were observed between the dugong and Steller's sea cow, 19 between the dugong and West Indian manatee, and 17 between the Steller's sea cow and the West Indian manatee (table 5.2). Between the dugong and Steller's sea cow, most of the replacements were observed at codons known to be highly variable within the cytochrome *b* gene. In contrast, between the dugong and manatee, replacements were observed at the more conservative codons (table 5.3 & figure 5.2; Irwin *et al.*, 1991). Any substitutions observed at the second position of a codon among any of the sirenian sequences resulted in a replacement (table 5.2).

Table 5.3: Frequency of replacements in the various sections of the cytochrome *b* gene. Replacements observed between the dugong (Irwin & Arnason, 1994), Steller's sea cow, and West Indian manatee (Ozawa *et al.*, 1997).

Section of the mitochondrial membrane	*Dugong vs Steller's sea cow*		*Dugong vs West Indian manatee*		*West Indian manatee vs Steller's sea cow*	
	level of conservatism for replacement					
	low	*high*	*low*	*high*	*low*	*high*
Inner surface	5	2	3	2	6	1
Transmembrane	2	1	2	5	4	3
Outer surface	3	1	3	3	2	1
total	**10**	**4**	**8**	**10**	**12**	**5**

5.3.3 Comparison of larger and smaller segments of the cytochrome *b* gene

Of the 225 bases of the cytochrome *b* gene compared between the dugong (Irwin & Arnason, 1994) and the West Indian manatee (Bradley *et al.*, 1993) (figure 5.4), no second position substitutions were observed, thus substitution rates and divergences times were estimated from the first and third positions only. Similarly, only one substitution (which was also a replacement) at the second position was observed in addition to changes at the first and third position, for the comparison between the dugong and Steller's sea cow, and for the comparison between the Steller's sea cow and West Indian manatee.

Table 5.4: Substitution rates estimated from a pairwise comparison among short and long segments of sirenian cytochrome *b* sequences. Sequence divergence was estimated using PHYLIP with the Kimura two-parameter algorithm (Kimura, 1980). Substitution rates were calculated considering divergence times (with upper and lower divergences shown within parentheses) proposed from immunological (Rainey *et al.*, 1984) and fossil (Domning, 1994) data. Substitution rates were estimated from replacement (R) substitutions at first and second position (1st & 2nd). Assuming a ts:tv ratio of 10:1, rates were estimated for substitutions (ts & tv) at 1st & 2nd position, and substitutions at third (ts & tv at 3rd). Assuming transversions (tv) occur in a 'clock-like' fashion, substitution rates were estimated from tv at all bases among all sites (total tv), and for substitutions at the 3rd position only (ts & tv at 3rd).

	Rate as % per Myr (range)					
	Dugong* vs *Steller's sea cow		***Dugong* vs *West Indian manatee***		***Steller's sea cow* vs *West Indian manatee***	
	Immunological 6 (4-8) Myr	Fossil >25 Myr	Immunological 18.5 (17-20) Myr	Fossil 35 (30-40) Myr	Immunological 18.5 (17-20) Myr	Fossil 35 (30-40) Myr
total tv						
225 bp	0.03 (0.05-0.03)	0.01	0.06 (0.07-0.06)	0.03 (0.04-0.03)	0.07 (0.08-0.07)	0.04 (0.04-0.03)
1005 bp	0.20 (0.30-0.15)	0.05	0.08 (0.09-0.08)	0 04 (0.05-0.04)	0.09 (0.10-0.08)	0.05 (0.05-0.04)
R sub at 1st & 2nd						
225 bp	0.25 (0.38-0 19)	0 06	0.05 (0.06-0.05)	0.03 (0.03-0.03)	0.05 (0.06-0.05)	0.03 (0.03-0.03)
1005 bp	0.23 (0 35-0.18)	0.06	0.08 (0.09-0.08)	0.04 (0.05-0.04)	0.08 (0.09-0.08)	0.04 (0.05-0.04)
ts & tv at 1st & 2nd						
225 bp	0.41 (0.60-0.30)	0.10	0.07 (0.08-0.07)	0.04 (0.05-0.03)	0.13 (0.14-0.12)	0.07 (0.08-0.06)
1005 bp	0.40 (0.60-0.30)	**0.10**	0.12 (0.13-0.11)	**0.06** (0.08-0.06)	0.13 (0.15-0.12)	**0.07** (0.08-0.06)
tv at 3rd						
225 bp	0.11 (0.17-0.08)	0.03	0.18 (0.20-0.17)	0.10 (0.11-0.08)	0.26 (0.28-0.24)	0.11 (0.13-0.10)
1005 bp	0.52 (0.78-0.39)	0.13	0.23 (0.25-0.22)	0.12 (0.14-0.11)	0.22 (0.24-0.20)	0.14 (0.16-0.12)
ts & tv at 3rd						
225 bp	4.00 (6.01-3.00)	1.00	1.19 (1.30-1 10)	0.63 (0.74-0.55)	2.80 (3.04-2.59)	1.48 (1.72-1.29)
1005 bp	4.94 (7.41-3.71)	1.19	1.52 (1.65-1.41)	0.82 (0.94-0.70)	1.77 (1.93-1.64)	0.94 (1.09-0.82)

A range of substitution rates were estimated assuming a divergence time obtained from immunological data (Rainey *et al.*, 1984) and the fossil record (Domning, 1994) (table 5.4). For example, the net nucleotide divergence (substitutions per site) between the shorter segment (i.e., 225 bases) of the dugong and West Indian manatee, for transitions and transversions at the first position (and second) as calculated under the Kimura two-parameter model (Kimura, 1980), was 0.0273. Assuming a divergence time of 18.5 Myr (37 Myr for two lineages) based on the average from immunological evidence (Rainey *et al.*, 1984), the predicted substitution rate was:

$$0.0273 \div 37 \text{ Myr} = 0.0007, \text{ or } 0.07\% \text{ per Myr (table 5.4).}$$

Alternatively, the coalescence times for the two sequences (species) were estimated from the net nucleotide divergence assuming a rate of substitution. For example, assuming a substitution rate of 0.11% per Myr (i.e., $0.0011 \div 2 = 0.00055$ substitutions every Myr for 2 x lineages) for the total number of transversions (Ozawa *et al.*, 1997), with a net nucleotide divergence between the 1005 bp of the dugong and West Indian manatee of 0.0239, the predicted divergence time was:

$$(0.0239 \div 0.00055) \div 2 \text{ (divergence between 2 lineages)} = 22 \text{ Myr (table 5.4).}$$

The shorter segment of the cytochrome *b* provided substitution rates that were generally slower than those estimated from their longer counterparts (table 5.4). Examination of table 5.4 indicates substitutions at the first and second position provided the most consistent estimates of substitution rates irrespective of the length. Using substitutions at the 1st and 2nd positions (table 5.4), the longer segment, and fossil divergence times only (as they are deemed the more accurate), an average substitution rate of 0.15% per Myr per lineage was obtained (i.e., data in bold type in table 5.4). This substitution rate was considered when predicting divergence times among sirenians as summarised in table 5.5. The most inconsistent estimates of substitution rates were provided by 'substitutions at the third position' despite assuming a ts:tv ratio of 10:1 (table 5.4).

Any divergence time more recent than 10 Myr among the sirenians (table 5.5), is likely to be a gross underestimate considering the fossil evidence (Domning, 1994). This suggests that the various mammalian substitution rates presented by Irwin *et al.* (1991) are inappropriate for sirenians (table 5.4). The rate of 0.11% per Myr per lineage for all

transversions provided by Ozawa *et al.* (1997) is expected to be more accurate as it is in better agreement with the fossil evidence (table 5.4). However, considering the consistency of substitution rates provided using transitions and transversion at the first and second position, with an assumed 10:1 ts:tv ratio, the substitution rate of 0.15% per Myr is probably the most accurate estimate for shorter segments of the cytochrome *b* gene (i.e., 225 bp), and as a consequence, is also likely to provide an accurate prediction of divergence time among sirenians.

Table 5.5: Coalescence times between sirenian sequences estimated from the shorter (225 bp) and longer (1005 bp) segment of the cytochrome *b* gene. Time of divergence between two sequences was predicted assuming the various substitution rates indicated by the references. Note that the published rates are substitution rates per lineage, rather than substitution rate *per se*.

Species compared	*Reference*	*Time in Myr*	
		225 bp	*1005 bp*
Dugong	0.11%/Myr total tv (Ozawa *et al.*, 1997)	4	22
vs	0.4%/Myr R at 1st & 2nd (Irwin *et al.*, 1991)	8	7
Steller's sea cow	0.5%/Myr tv at 3rd (Irwin *et al.*, 1991)	3	13
	10%/Myr ts & tv at 3rd (Irwin *et al.*, 1991)	5	6
	0.15%/Myr sub at 1st & 2nd (table 5.4)	32	32
Dugong	0.11%/Myr total tv (Ozawa *et al.*, 1997)	20	28
vs	0.4%/Myr R at 1st & 2nd (Irwin *et al.*, 1991)	5	8
West Indian manatee	0.5%/Myr tv at 3rd (Irwin *et al.*, 1991)	13	17
	10%/Myr ts & tv at 3rd (Irwin *et al.*, 1991)	4	6
	0.15%/Myr sub at 1st & 2nd (table 5.4)	18	30
Steller's sea cow	0.11%/Myr total tv (Ozawa *et al.*, 1997)	24	30
vs	0.4%/Myr R at 1st & 2nd (Irwin *et al.*, 1991)	5	7
West Indian manatee	0.5%/Myr tv at 3rd (Irwin *et al.*, 1991)	16	19
	10%/Myr ts & tv at 3rd (Irwin *et al.*, 1991)	10	7
	0.15%/Myr sub at 1st & 2nd (table 5.4)	32	33

5.4 DISCUSSION

5.4.1 Sequence characteristics

The dugong cytochrome *b* gene shares sequence characteristics with 17 ungulate and cetacean cytochrome *b* genes (Irwin *et al.*, 1991; Irwin & Arnason, 1994). The cytochrome *b* gene sequence begins with a conserved initiating methionine (M) codon (ATG), and is composed of a total of 379 codons (380 if you include the stop codon).

Despite the close phylogenetic relationship suggested by molecular (de Jong *et al.*, 1981; Rainey *et al.*, 1984; Barriel *et al.*, 1993; Springer & Kirsch, 1993; Irwin & Arnason, 1994; Lavergne *et al.*, 1996; Porter *et al.*, 1996; Stanhope *et al.*, 1996; Ozawa *et al.*, 1997) and morphological (Domning *et al.*, 1986; Novacek *et al.*, 1988; Novacek, 1990) data, sirenians do not share the high rate of evolution of the cytochrome *b* gene of proboscideans (Irwin *et al.*, 1991; Ozawa *et al.*, 1997). Furthermore, sirenians do not have some of the unusual sequence characteristics of the African elephant (*Loxodonta africana*), which includes an amino acid insertion (TCC) between cytochrome *b* codon positions 325 and 326, a deletion observed at the 3' end (i.e., codon 380), and a termination with TAA rather than an AGG stop codon (Irwin *et al.*, 1991). The base composition bias of the cytochrome *b* gene of the dugong was concordant with that of the pig (*Sus scrofa*), peccary (*Tayassu tajacu*), perissodactyls, rodents (*Rattus norvegicus* and *Mus domesticus*), African elephant (*Loxodonta africana*) and human (*Homo sapiens*) (Irwin *et al.*, 1991), in that approximately 50% of the alanine codons among the sirenian sequences (figure 5.4) were GCC.

The observed variation of the cytochrome *b* gene of the Torres Strait and Exmouth Bay dugongs (samples T677 and WA respectively) in relation to the published dugong sequence (Irwin & Arnason, 1994), is possibly real for site 225 (figure 5.4). It is a silent substitution at the third codon position. This type of difference is characteristic of intraspecific mutation observed for the cytochrome *b* gene (e.g., Lento *et al.*, 1994). The replacement substitution at site 386 is not improbable of intraspecific variation. However, considering the paucity of substitutions at the third position, a replacement at the second position of a codon is unusual. Furthermore, this substitution is characteristic of *Taq* polymerase error, namely pyrimidine (CT) transitions which occur at a higher frequency (figure 5.5). For these reasons, I consider

that the substitution at site 386 is a PCR artefact. The last variation (site 1022) is uncharacteristic of real coding sequences. The occurrence of an insertion is highly unlikely as this would interrupt the gene function. Frameshifts (i.e., an insertion) occur at a rate of one base per 41,000 (Tindall & Kunkel, 1988). Only one clone for the dugong cytochrome *b* samples was sequenced, therefore I am unable to confirm if the variations are real or artefacts.

The observed number of nucleotide differences between the dugong clones and the published dugong cytochrome *b* sequence was 2.6×10^{-4} per base per cycle (Irwin & Arnason, 1994), which is within the range of expected error from *Taq* polymerase (Saiki *et al.*, 1988; Tindall & Kunkel, 1988; Keohavong & Thilly, 1989).

As there was apparently only one 'real' substitution among the 1085 bases of dugong sequence compared, it seems the cytochrome *b* is not sufficiently variable for a population level study, which is why I did not investigate it further. This conclusion is further supported by the presence of only one transition among the two published West Indian manatee sequences (figure 5.4).

5.4.2 The structure-function model for cytochrome *b*

The dugong cytochrome *b* gene does not challenge the current structure-function model of this membrane-spanning protein (section 5.1.1; Irwin & Arnason, 1994). Of the sirenian cytochrome *b* sequences (Irwin & Arnason, 1994; Ozawa *et al.*, 1997), the most frequently occurring replacements in the transmembrane region were exchanges between the hydrophobic amino acids isoleucine (I) and valine (V), as predicted (figure 5.3 and figure 5.4; Irwin *et al.*, 1991).

5.4.3 Substitution rates among sirenians

Ozawa *et al.* (1997) estimated a substitution rate of 0.11% per Myr per lineage among sirenians based on transversions in cytochrome *b* gene sequences. This contrasts with the faster substitution rate for proboscideans as suggested by Irwin *et al.* (1991), and predicted to be 0.30% per Myr per lineage by Ozawa *et al.* (1997). Therefore it is inappropriate to calculate divergence times among sirenians using the substitution rates of proboscideans. It is possible that rate variation also exists among sirenians. This could only be

addressed if sequences were obtained from the other manatee species (*Trichechus inunguis* and *T. senegalensis*) (Ozawa *et al.*, 1997).

The estimates for substitution rates in the cytochrome *b* gene using the shorter segment of cytochrome *b* (Bradley *et al.*, 1993) were generally lower than the estimates from the longer segment (table 5.4). The lower substitution rates are a result of the smaller divergences among the sirenian sequences. In conclusion, a short segment of the gene sequence can provide a reasonable estimate of substitution rates, but if incorrect for sirenians, will underestimate rather than overestimate the rate. Substitution rates are likely to be more accurate if based on a larger portion of the gene.

5.4.4 Time of divergence among sirenians

Edwards *et al.* (1991) found that using the whole cytochrome *b* gene, rather than the portion (about 300 bases) amplified using primers designed by Kocher *et al.* (1989), more than trebled the 'phylogenetic resolving power'. The comparisons I made with a shorter (225 bases) and a longer (1005 bases) segment of the cytochrome *b*, generally support this conclusion. However, the consistency of the substitution rate estimates from the first and second position, relative to the other estimates in table 5.4, suggests the selection of the codon position and type of substitution are more important than segment length *per se*.

The similarity in divergence times suggested among the dugong, West Indian manatee and Steller's sea cow is surprising. The cytochrome *b* sequences indicate that these sirenians diverged around 25 to 35 Myr. The cytochrome *b* data do not challenge the topology of the morphological tree (i.e., there is no dispute that the dugong and Steller's sea cow are sister species compared to the manatee) (Ozawa *et al.*, 1997). However, the coalescence times for these sirenians may be much closer than proposed from the fossil data (Savage, 1976; Domning, 1994; Domning & Hayek, 1986). Fossil data indicate a common ancestor no later than upper Oligocene for the dugong and Steller's sea cow (Domning, 1994), and a divergence between the dugongids and trichechids perhaps in the lower Oligocene, but more likely in the upper Eocene (Domning, 1994). The cytochrome *b* gene data suggest that all three sirenians diverged in the Oligocene (Ozawa *et al.*, 1997), which is not completely refuted by the fossil data (Domning, 1994). Alternatively, the closeness of the estimates of divergence times from

cytochrome *b* data may be explained by variation in substitution rate among sirenians.

5.5 CONCLUSIONS

A length of 225 bp of the cytochrome *b* gene is able to approximate the rates predicted by 1005 bp of the same gene. However, selection of the codon position and type of substitution appear to be more important than segment length in the estimation of substitution rates. Other than substitution rates for the cytochrome *b* gene, sirenians display the mutation spectrum typical of most mammals including: 1) a higher transition to transversion ratio that decreases with the more distantly related sirenians, 2) replacements at the more variable amino acid residues between the closely related sirenians (dugong and Steller's sea cow), and 3) the frequency of replacements in the various sections of the mitochondrial membrane. This suggests that assumptions applicable to most mammals regarding structural features of the cytochrome *b* can be confidently extended to include the dugong. A substitution rate for first and second positions assuming a ts:tv ratio of 10:1 of 0.15% per Myr per lineage is proposed for the cytochrome *b* gene.

Only three differences were observed between the 1085 bp dugong sequences, only one of which was considered as 'real', indicating that the cytochrome *b* gene is not sufficiently variable for an intraspecific level study of the dugong. Subsequently, the control region of mtDNA, renowned for its higher level of variation, was selected to resolve within-species differentiation of the dugong (Chapters 6 & 7).

CHAPTER SIX

CHARACTERISATION OF THE CONTROL REGION

The cytochrome *b* gene does not have the level of nucleotide variation required for an intraspecific study of the dugong (Chapter 5). For this reason, the control region of mtDNA was investigated (this chapter). Characterisation of the control region revealed a hypervariable portion positioned 5′ in the control region, which was then used to examine intraspecific genetic structure of the dugong (Chapter 7).

6.1 INTRODUCTION

6.1.1 Suitability of the control region for intraspecific studies

The control region of mtDNA which includes the displacement (D) loop (figure 5.1), is the most variable portion of the mitochondrial genome with respect to sequence and length (Upholt & Dawid, 1977; Walberg & Clayton, 1981; see Harrison (1989) for a review). In the D-loop, the rate of mutation has been estimated to be five to ten times that of the rest of the mitochondrial genome (Aquadro & Greenberg, 1983; Greenberg *et al.*, 1983; Brown *et al.*, 1993). Comprising between 5% and 7% (800-1200 bases) of the mammalian mitochondrial genome, the control region spans the segment between the phenylalanine (Phe–) and proline (Pro–) transfer RNA (tRNA) genes (figures 5.1 & 6.1) and is the only major non coding region in mtDNA. The control region consists of three domains: 1) a left domain (5' end), where during mtDNA replication the nascent heavy (H) strand pauses and it is determined whether synthesis is arrested or continued, 2) a conserved central domain (approximately 200 bases long) and, 3) a hypervariable right domain (3' end) containing the origin of H-strand replication adjacent to Phe-tRNA (Greenberg *et al.*, 1983; Saccone *et al.*, 1991).

The central domain is reported to be conserved in all mammals investigated so far and is easily aligned between species (Bibb *et al.*, 1981; Anderson *et al.*, 1981, 1982; Mignotte *et al.*, 1987; Southern *et al.*, 1988;

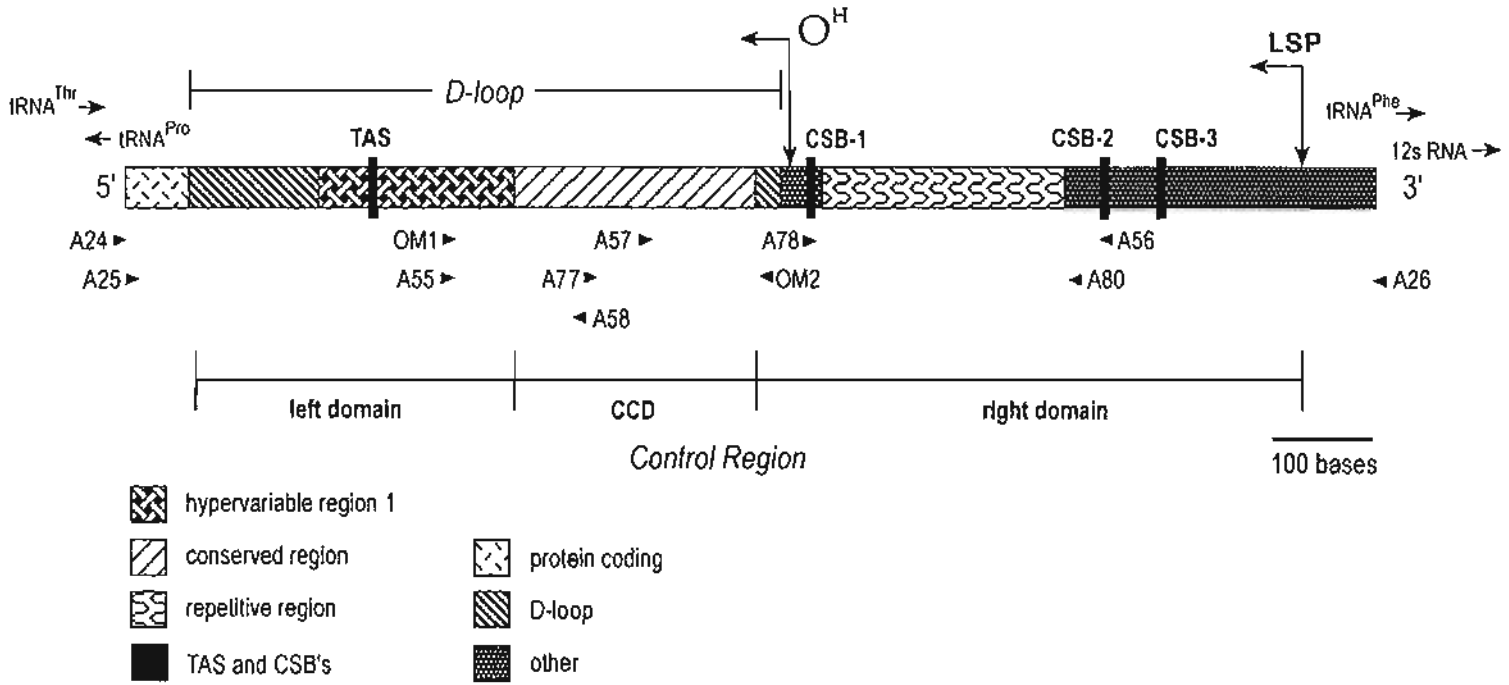

Figure 6.1: Schematic diagram of the control region sequence of mitochondrial DNA from the dugong as detailed in figure 6.2. The sequence orientation is shown reading 5' to 3' on the L-strand. The strand containing the sense sequence for the primers and the protein coding genes is denoted by ——→ (L-strand) or ←—— (H-strand). Primers used to amplify and sequence the control region are: A24, A25, A26, A55, A56, A57, A58, A77, A78, A80, OM1 & OM2 (see table 4.1). Genes flanking the D-loop are the transfer RNA's: Threonine, Proline, Phenylalanine and the ribosomal 12s RNA. O$_H$ designates the origin of DNA replication for the H-strand. LSP refers to L-strand promoter. TAS refers to Termination Associated Sequence, CSB to Conserved Small Block, and CCD to Central Conserved Domain, of which the sequences are detailed in figures 6.4 & 6.5 respectively.

Hoelzel *et al.*, 1991; Saccone *et al.*, 1991). Within this domain, a putative weakly conserved ORF (open reading frame) has been proposed, containing a highly conserved motif potentially encoding seven amino acids (Saccone *et al.*, 1987; Hoelzel *et al.*, 1991). However, the left and right domains of the control region evolve rapidly, generating variation in both sequence and length composition. These areas are prone to insertion and deletion of elements and the generation of repetitive DNA (particularly at the 3' end) via replication slippage (Greenberg *et al.*, 1983; Saccone *et al.*, 1991).

Sequences in the left domain rich in AT are called Termination Associated Sequences (TAS elements), and may be signals for the termination of D-loop synthesis (Doda *et al.*, 1981; Brown *et al.*, 1986). Often more than one TAS element can be recognised among control region sequences of various vertebrate species, however they are not always present at the same location (Brown *et al.*, 1986).

Three small blocks conserved among species, CSB-1, CSB-2 and CSB-3, exist outside of the conserved central domain (i.e., in the right domain), and are positioned sequentially between the origin of the H-strand replication and the L-strand promoter (Saccone *et al.*, 1991). Sometimes CSB-2 and CSB-3 are not recognised as separate entities and another version, CSB 2+3 can be recognised. CSB 2+3 is believed to have evolved first (Saccone *et al.*, 1991; Ghivizzani *et al.*, 1993). Most artiodactyl species and cetaceans have the CSB-1 and CSB 2+3 series of conserved blocks (Southern *et al.*, 1988; Arnason *et al.*, 1991; Saccone *et al.*, 1991).

Despite the high level of sequence diversity among mammalian species in sequence and length in the left and right domains of the control region, the presence of small conserved blocks of sequence and the (sometimes conserved) positioning of cloverleafs in these domains, suggests a common function. That is, they support the idea of a regulatory mechanism for the control region in mtDNA (Saccone *et al.*, 1991). It appears that in the control region, evolutionary pressure is active not only at the level of sequence composition, but as well as, or perhaps more so, at the secondary structure level (Brown *et al.*, 1986; Mignotte *et al.*, 1987).

The objectives of this chapter are to: 1) determine if the control region of the dugong is sufficiently variable to recognise intraspecific subdivision, and

2) assess whether features of the control region characteristic among other mammalian taxa, are also shared with the dugong.

6.2 METHODS

Torres Strait and Moreton Bay (figure 3.2) are separated by approximately 2600 km of coastline, much of which is suitable habitat for the dugong. DNA was extracted and purified from an individual dugong from each of these populations (samples T677 (Torres Strait) and D3 (Moreton Bay)) following section 4.1. The control region of mtDNA was initially amplified (section 4.3) using the primers designed by Kocher *et al.* (1989), cloned (section 4.5), and the ends of the control region sequenced (section 4.4). Dugong specific primers were then designed (section 4.3.1) in order to sequence the remaining internal portions of the control region (figure 6.2). The primer sequences are listed in table 4.1, with their positions relative to the control region indicated in figure 6.1. The 5' end of the control region was similarly sequenced, both directly and indirectly, forwards and backwards, for a Florida manatee specimen (Appendix C), which was subsequently used as an outgroup.

Two clones from each individual (i.e., T677 and D3) were sequenced. If a discrepancy existed between them (because of *Taq* polymerase error, section 5.2.1), that portion of the control region was sequenced in a third clone, or the PCR product sequenced directly (section 4.4) using the dugong-specific internal primers. Furthermore, the primers were used to sequence 193 bases (5' end of the control region) of a Florida manatee.

The control region sequences of a Moreton Bay (sample D3) and Torres Strait (sample T677) dugong were aligned (section 4.7) to each other, as well as to other vertebrate species to confirm the sequence: 1) as D-loop (by aligning the more conserved blocks of sequence), 2) as dugong (i.e., to exclude the most likely sources of contamination: bacteria, yeast, human), and 3) to characterise conserved and variable region(s). MacClade (Macintosh, Version 3; Maddison & Maddison, 1992) was used to generate sequence statistics.

The control region sequences of other species aligned with the dugong were obtained from a genome library facility, NCBI (National Center for

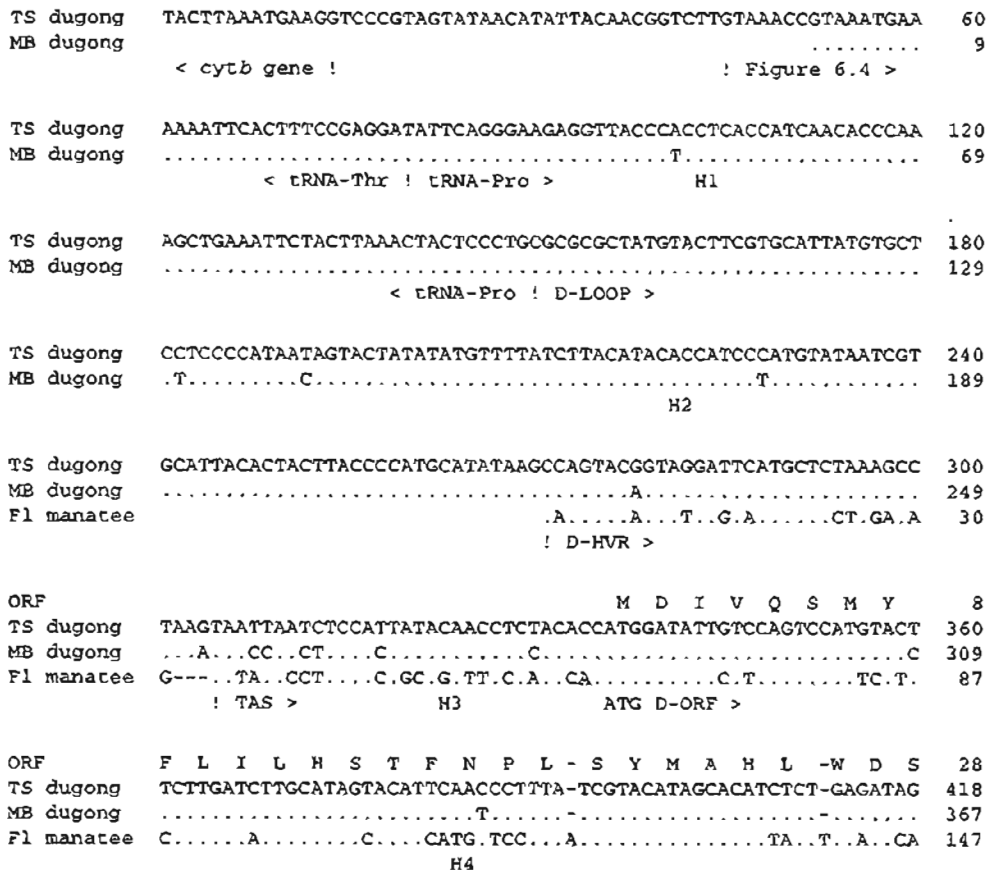

Figure 6.2: Alignment of dugong and Florida manatee control regions. The sequence orientation is shown reading 5' to 3' on the L-strand. The aligned control region sequences include: a Torres Strait (TS, sample T677) and a Moreton Bay (MB, sample D3) dugong, and a Florida manatee (Fl manatee). Hyphens indicate gaps in the sequence, a period indicates sites where the MB dugong is the same as the TS dugong sequence. Differences to the reference sequence are indicated by the variant base. Exclamation marks indicate a feature nearby primarily related to the dugong sequences. < and > indicate the direction in which the feature is positioned. D-HVR refers to hypervariable region 1 in the dugong. CCDOM refers to the conserved central domain. CSB-1, CSB-2 & CSB-3 indicate the minor conserved blocks. O-H is the origin of H-strand replication, LSP is the L-strand promoter (as underlined), located on the dugong sequence by matching with human, porcine and bovine LSP sequences (Saccone *et al.*, 1991; Ghivizzani *et al.*, 1993). The translated region represents the putative ORF which begins with a conserved methionine (M), the amino acids are detailed in table 5.1. C–ORF refers to the conserved seven (putative) amino acids as underlined. Other underlined sequences refer to the conserved blocks of DNA as detailed in figures 6.4 and 6.5. H1 to H17 indicate the placement of hairpins in the secondary structure of the control region as shown in figure 6.6. Numbers at the end of the sequence indicate the nucleotide number, omitting gaps, markers and repetitive DNA. With respect to the ORF sequence, numbers at the end of the sequence refer to the amino acids rather than nucleotides. This figure continues for an additional page.

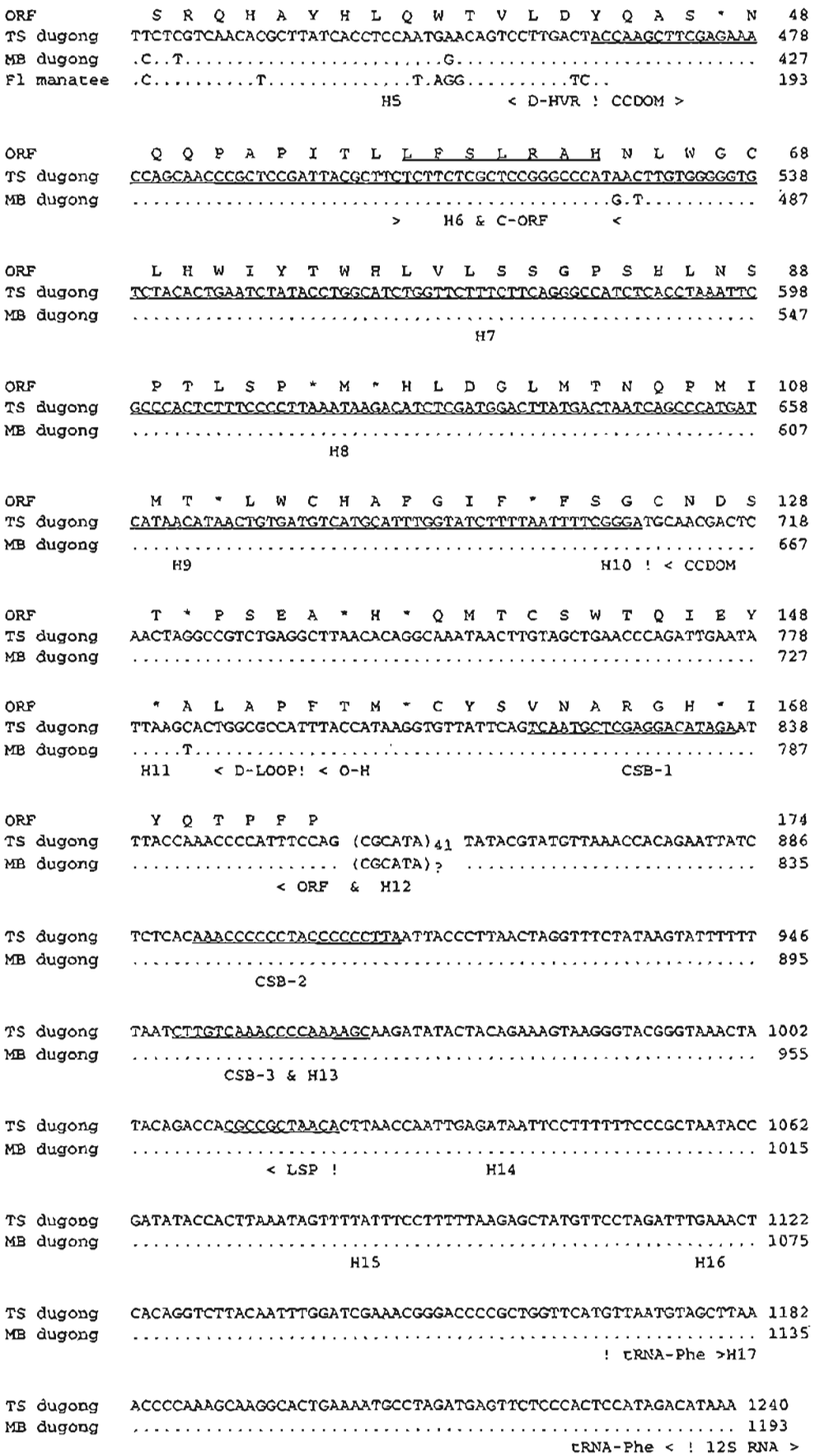

Figure 6.2: Continued.

Biotechnology Information, http://www2.ncbi.nih.gov/cgi-bin/genbank) available on the Internet.

Secondary structure programs were also accessed from the Internet. The program 'RNA 2° structure' (in 'GCG') which was appropriate for single-stranded DNA, was used to test the ability of the mtDNA control region sequence to fold onto itself. The graphics program 'Squiggles' was then used to form the illustration. Both of these programs are in ANGIS (The Australian National Genomic Information Service). Only the most optimal structure is produced by this program.

The secondary structures for the shorter segment of the control region were constructed using MFOLD (version 2.3) available at the Internet address: http://www.ibc.wustl.edu/~zuker/dna/form1.cgi In MFOLD, 15% suboptimality restrictions were exerted on the sequence, and a range of structures were produced with measurements of free energy indicating optimality. The smaller the free energy, the more optimal the structure. The free energy measurements (provided by both the secondary structure programs) were not comparable unless the DNA sequences were of the same length.

6.3 RESULTS

Approximately 1190 bases (excluding the repeat region) were sequenced and aligned between the Moreton Bay and Torres Strait dugongs, and 193 bases of a Florida manatee. The distance between the dugong and Florida manatee haplotypes (using the Tamura-Nei corrected distances, Appendix F) ranged from 27% (H#33) to 61% (H#3). Of the 19 nucleotide substitutions between the two dugong sequences, 16 (84%) occurred 5' of the central conserved block, compared to two (11%) within the 155 bases of the central conserved block, and one (5%) 3' of the central conserved block (figure 6.2). There were no transversions observed in the entire control region compared between the two dugong sequences (i.e., all of the substitutions were transitions). The base guanine (G) persisted with low frequency relative to the other nucleotides, throughout all three domains of the control region (figure 6.3). The DNA sequence 5' of the central conserved block has been nominated as 'hypervariable region 1' in humans by

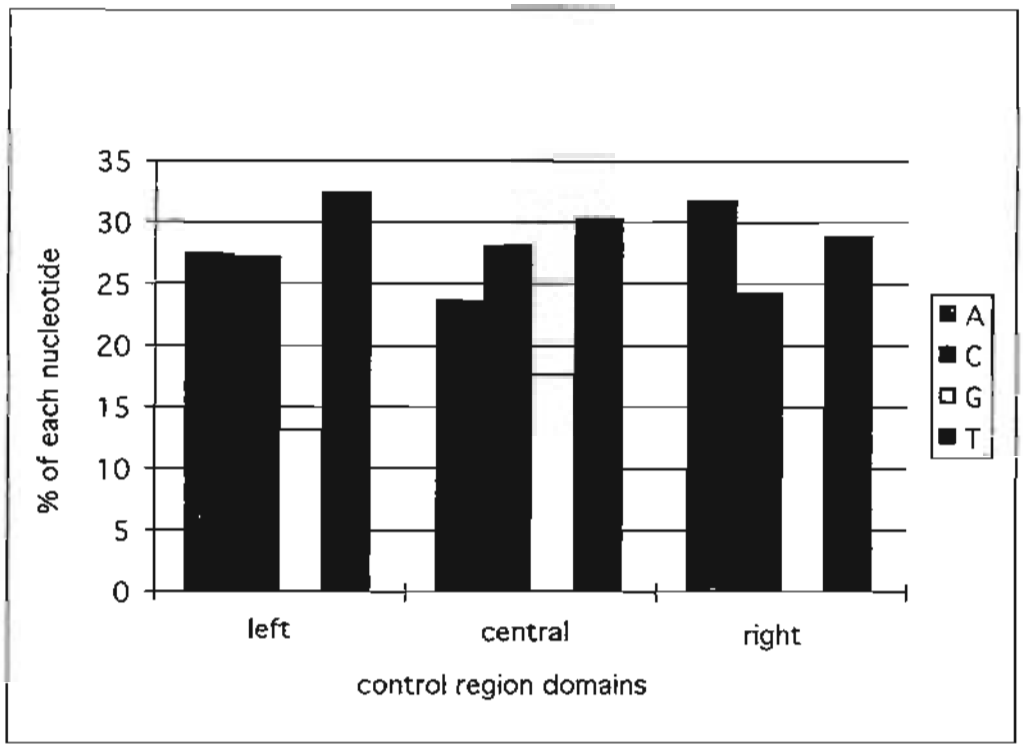

Figure 6.3: The frequency of each nucleotide within the control region domains.

TAS

```
TS Dugong     00305 TAATTAATCTCCATTAT 00321
MB Dugong     00254 ...CC..CT....C... 00270
Fl Manatee    00032 ..TA..CCT....C.GC 00048
Armadillo     15767 ..CA.T-.A..T.CCCC 15782
Rhinoceros    00374 ..CA.A..ACAT..... 00390
Horse         15652 ..CA.T.---TAT.AT. 15665
Bovine        16007 ..CA.T.AA-TT..A-. 16021
Blue whale    16016 ..CA..-..A-...A.. 16130
Chimpanzee    15734 ..CA..-CAA-TCAC.C 15748
Human         16157 ..CA...-AAA.CCA.. 16172
Seal          16563 ..CA..TA.GG...ATA 16579
Cat           16738 ..TA..TA..A...A.- 16753
Mouse         15496 ..CA.T.-AA--TCA.. 15509
Rat           00047 ..CA.T.-A--AT.A-. 00059
Rabbit        00863 ..CA.C.CA--...A.. 00877
Platypus      15584 ..CA.T..A--TG.ATA 15598
Opossum       15630 ..CA.T..A.T...A.. 15646
```

CSB-2

```
TS Dugong     00893 AAACCCCCCTACCCCCCTTA 00913
MB Dugong     00842 ..................... 00862
Fl manatee          not sequenced
Armadillo     16444 .........CCT.....CCC 16463
Rhinoceros    01052 .........C-A......AT 01073
Horse         16396 .........CTA.....CAT 16417
Blue whale    00259 .........-T...T..A.. 00277
Chimpanzee    16291 .........CCT.....CGG 16310
Human         00300 .........C--T....CGC 00316
Seal          00662 .........-TTA....CCG 00680
Cat           00607 .........-TTA....CCG 00625
Mouse         16085 .........---A....C.C 16101
Rat           00667 .........C--A....C.G 00684
Rabbit        01720 .........C-TA....CCC 01738
Platypus      16137 --C......CTT.....CCC 16154
Opossum       16627 ...--....CTTA....C.. 16644
```

CSB-1

```
TS Dugong     00817 TCAATGCTCGA-GGACA-TAGA 00836
MB Dugong     00766 ..........-.....-.... 00785
Fl manatee          not sequenced
Armadillo     16241 ......G.TACA.....-..A. 16261
Rhinoceros    00824 ......G.TACA.....-..C. 00844
Horse         16035 .T..ATTGAAGCT.GACT..A. 16056
Bovine        00182 ......G..ACA.....-..A. 00202
Blue whale    00190 .T....G.TACA.....-...T 00210
Chimpanzee    16210 .T......T.TA.....-..AC 16230
Human         00216 .T......T.TA.....-..AT 00236
Seal          00190 ......G.A.CG.....-...T 00210
Cat           00194 ......G..ACA.....-..T. 00214
Mouse         16035 .TC.....T.TTA....-..A. 16055
Rat           00617 .TC...T.T.TAA....-..A. 00637
Rabbit        01394 .T......T.TC.....-..A. 01414
Platypus      16107 .T......T..C.....-..A. 16127
Opossum       16284 .T...ATA...A.....A..A. 16305
```

CSB-3

```
TS Dugong     00951 CTTGTCAAACCCCAAAAGC 00969
MB Dugong     00900 ..................... 00918
Fl manatee          not sequenced
Armadillo     16498 ................A. 16516
Rhinoceros    01106 ...C............A. 01124
Horse         16448 ....C...........A. 16466
Blue whale    00302 T.CAC..CC....T.G.CA 00320
Chimpanzee    16331 TC..C...........A. 16349
Human         00344 TC..C...........A. 00362
Seal          00717 TC..C...........A. 00735
Cat           00662 ....C.........C..A. 00680
Mouse         16109 --..C.......A....A. 16125
Rat           00710 --..C...........A. 00726
Rabbit        01791 .C..C...........A. 01809
Opossum       16667 TCC.............C. 16685
```

CSB-2+3

```
Platypus      16137 CCCCCCCCTTCCCCCCCCAAAAAATTTTTT- 16166
Cow           00216 --.........------AT........CCCC 00238
```

Figure 6.4: Aligned TAS and CSB sequences (adapted from Gemmell *et al.*, 1996). Alignment of mammalian termination-associated sequences (TAS) and conserved sequence blocks (CSB) in the control region of mtDNA. The numbers at the ends of each sequence indicate nucleotide position omitting gaps, as cited in GENBANK. The accession number, species name and reference of the mammals compared are detailed as follows: platypus (X83427, *Ornithorhynchus anatinus*, Janke *et al.*, 1996), opossum (Z29573, *Didelphis virginiana*, Janke *et al.*, 1994), mouse (J01420, *Mus musculus*, Bibb *et al.*, 1981), Norway rat (X04733, *Rattus norvegicus*, Brown *et al.*, 1986), European rabbit (X54172, *Oryctolagus cuniculus*, Mignotte *et al.*, 1990), harbor seal (X63726, *Phoca vitulina*, Arnason & Johnsson, 1992), domestic cat (U20753, *Felis catus*, Lopez *et al.*, 1996), common chimpanzee (X93335, *Pan troglodytes*, Arnason *et al.*, 1996), human (J01415, *Homo sapiens*, Anderson *et al.*, 1981), black rhinoceros (L22010, *Diceros bicornis*, Jama *et al.*, 1993), horse (X79547, *Equus caballus*, Xu & Arnason, 1994), bovine (V00654, *Bos taurus*, Anderson *et al.*, 1982), blue whale (X72204, *Balaenoptera musculus*, Arnason *et al.*, 1993), and armadillo (Y11832, *Dasypus novemcinctus*, Arnason *et al.*, 1997). The Florida manatee (Fl manatee), Torres Strait and Moreton Bay dugong (TS and MB dugong respectively) sequences are from this study, and their nucleotide positions correspond to those in figure 6.2. A hyphen indicates a gap in the sequence, a period denotes common sequence. Differences to the reference sequence (i.e., TS dugong) are indicated by the variant base.

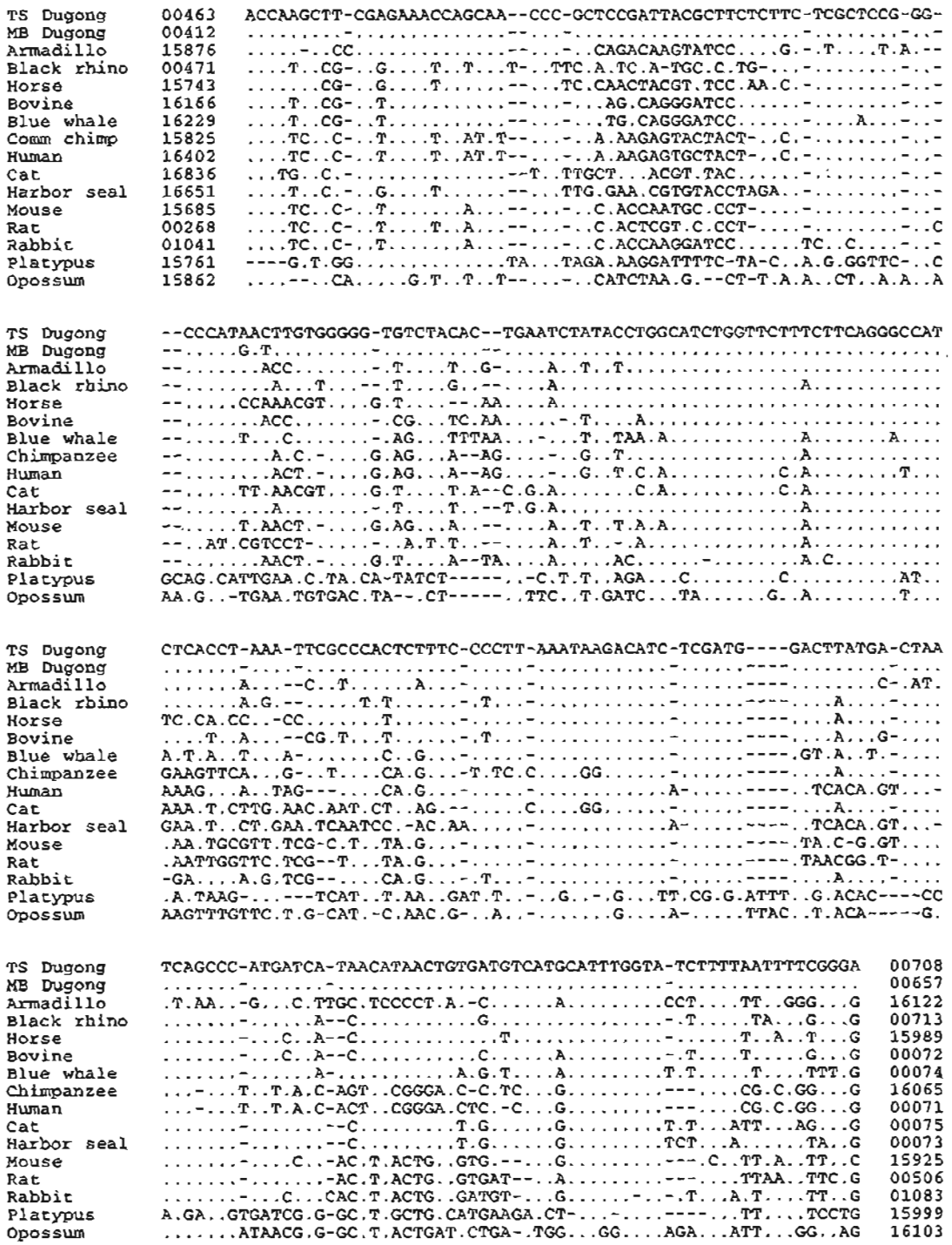

Figure 6.5: Central conserved domain (CCD). Alignment of the CCD among species (adapted from Gemmell *et al.*, 1996). See figure 6.2 for sequence details.

Wakeley (1993), and this region represents about 17% of the bases in the entire control region in the dugong.

6.3.1 The left domain (including hypervariable region 1)

In the dugong, the left domain included 313 nucleotides of the control region (figures 6.1 & 6.2). Of the differences observed between the Moreton Bay and Torres Strait dugong sequences, 81% occurred in the 194 bases of hypervariable region 1 (HVR-1). The most efficient primers for sequencing HVR-1 were A58 and A24 (refer to table 4.1 for primer sequence and figure 6.1 for the position of the primer in the control region). These primers were used routinely to amplify 194 bases of HVR-1 in an additional 101 dugongs, as well as in a Florida manatee (193 bases) and examined for intraspecific genetic structure in Chapter 7.

6.3.2 Conserved regions

6.3.2.1 The conserved central domain and smaller conserved blocks

The small conserved blocks, CSB-1, CSB-2, CSB-3 and termination-associated elements (TAS) were located by aligning common sequences between the dugong and other mammals (figure 6.4). The conserved block CSB-2+3, recognised in the cow (Anderson *et al.*, 1982) and platypus (Janke *et al.*, 1996) control regions, was not apparent in the dugong. A larger conserved block or 'conserved central domain' of the dugong control region was also located by aligning common sequences among mammals (figure 6.5). The L-strand transcriptional site (LSP) in the dugong was located by alignment with the LSP site of porcine and bovine sequences (figure 6.2; Saccone *et al.*, 1987; Ghivizzani *et al.*, 1993).

6.3.2.2 ORF's

A putative ORF (see glossary for definition) proposed by Saccone *et al.* (1987) was located in the dugong sequence by identifying similar sequence in other mammalian species (Saccone *et al.*, 1987; Hoelzel *et al.*, 1991) and a hypothetical translation is presented (figure 6.2). The seven amino acid motif LFSLRAH (see table 5.1 to translate the single letter code) is highly conserved among vertebrates (Saccone *et al.*, 1987; Hoelzel *et al.*, 1991) and was also found in the dugong (underlined text in figure 6.2). Because of substitutions between the Torres Strait and Moreton Bay dugong sequences, there would have been 'replacements' at codon positions 13 and 14, upstream of the motif LFSLRAH (figure 6.2). In the dugong, three potential

ATG (methionine, M) 'start sites' occurred upstream to this motif at amino acid positions: one, six and 22 in figure 6.2. However, an AGA termination codon (indicated by an asterisk in figure 6.2) 31 codons downstream of the conserved codon block (followed immediately downstream by an additional eight stop codons) is evidence against the existence of an ORF.

6.3.3 The right domain (including the repeat region)

Within the 464 bases (excluding the repeat motifs), only one substitution was observed in the 5' end of the control region. However, a microsatellite (i.e., a six base motif CGCATA) was found repeated in tandem in both of the Torres Strait and Moreton Bay dugongs. The microsatellite is positioned in the right domain, between CSB-1 and CSB-2 (i.e., 3' end of the control region, figures 6.1 & 6.2). In the Torres Strait dugong, the six base motif (CGCATA) is repeated uninterrupted in tandem 41 times. The exact size and nature of the repeat in the Moreton Bay dugong (control region clones) is uncertain, as I was unable to sequence all the way through the repeat in a single sequencing run. However, the repeat motifs continue for an undetermined number after a stretch of intervening sequence. This intervening sequence varied for the two Moreton Bay dugong clones sequenced, suggesting the presence of heteroplasmy:

Moreton Bay dugong (sample D3):

D3 (clone 10A): ...$(CGCATA)_?$ATAGCGCTATGCG$(CGCATA)_{42}$TATA...

D3 (clone 11A): ...$(CGCATA)_?$CACACA$(CGCATA)_3$CAGA$(CGCATA)_8$CACACACAG $(ATACGC)_2$ATACACACA$(CGCATA)_3$CACAGCGCA$(CGCATA)_?$?$(CGCATA)_{31}$TATA...

The microsatellite of the control region may have been informative as an additional molecular marker to examine intraspecific genetic structure in the dugong. It was investigated as such in Chapter 9.

6.3.4 Variation in base composition between dugong sequences

Of the 19 substitutions (see figure 5.3 for types of substitution) observed in the control region between the Torres Strait and Moreton Bay control region sequences (figure 6.2), MacClade (Maddison & Maddison, 1992) showed that 79% and 21% were CT and GA transitions respectively. Not one of the observed substitutions was a transversion (table 6.1). MacClade was also used to calculate the frequency of each base in the entire control region. The

base guanine (G) occurred at a lower frequency compared to the other bases as illustrated in figure 6.3, a characteristic that was consistent for HVR–1.

Table 6.1: Substitution frequencies. A total of 19 substitutions were observed between the aligned control region sequences of the Torres Strait (T677) and Moreton Bay (D3) dugongs. No transversions were observed.

	A	C	G	T
A				
C	0			
G	4	0		
T	0	15	0	

6.3.5 Secondary structure

Using the program RNA 2° structure, the entire control region sequenced from the Torres Strait dugong (sample T677), formed a number of cloverleaf structures (figure 6.6), yet none of these cloverleaf structures was specifically located in the TAS (Doda *et al.*, 1981) at the 5' end or within CSB-1 at the 3' end as discovered by Brown *et al.* (1986). However, a complex of cloverleaf structures was located downstream of the TAS elements and hairpin structures coincided with the repeat region, CSB-3 and Phe-tRNA (figure 6.6).

I was able to form a secondary structure comparable to those predicted by Brown *et al.* (1986) by using a conserved segment of the control region and the program MFOLD. MFOLD produced six structures from the conserved region CSB-1 plus flanking sequence, ranging in free energy between -4.4 and -5.3 kcal/mole, and using a 15% suboptimality. If additional sequence was included, the structures changed radically and failed to retain any conservatism of hairpins or cloverleafs among them (data not shown). The one structure that best approximated those predicted by Brown *et al.* (1986), had a free energy of -4.5 kcal/mole. This structure was modified slightly (figure 6.7), to improve its resemblance to the structures presented by Brown *et al.* (1986).

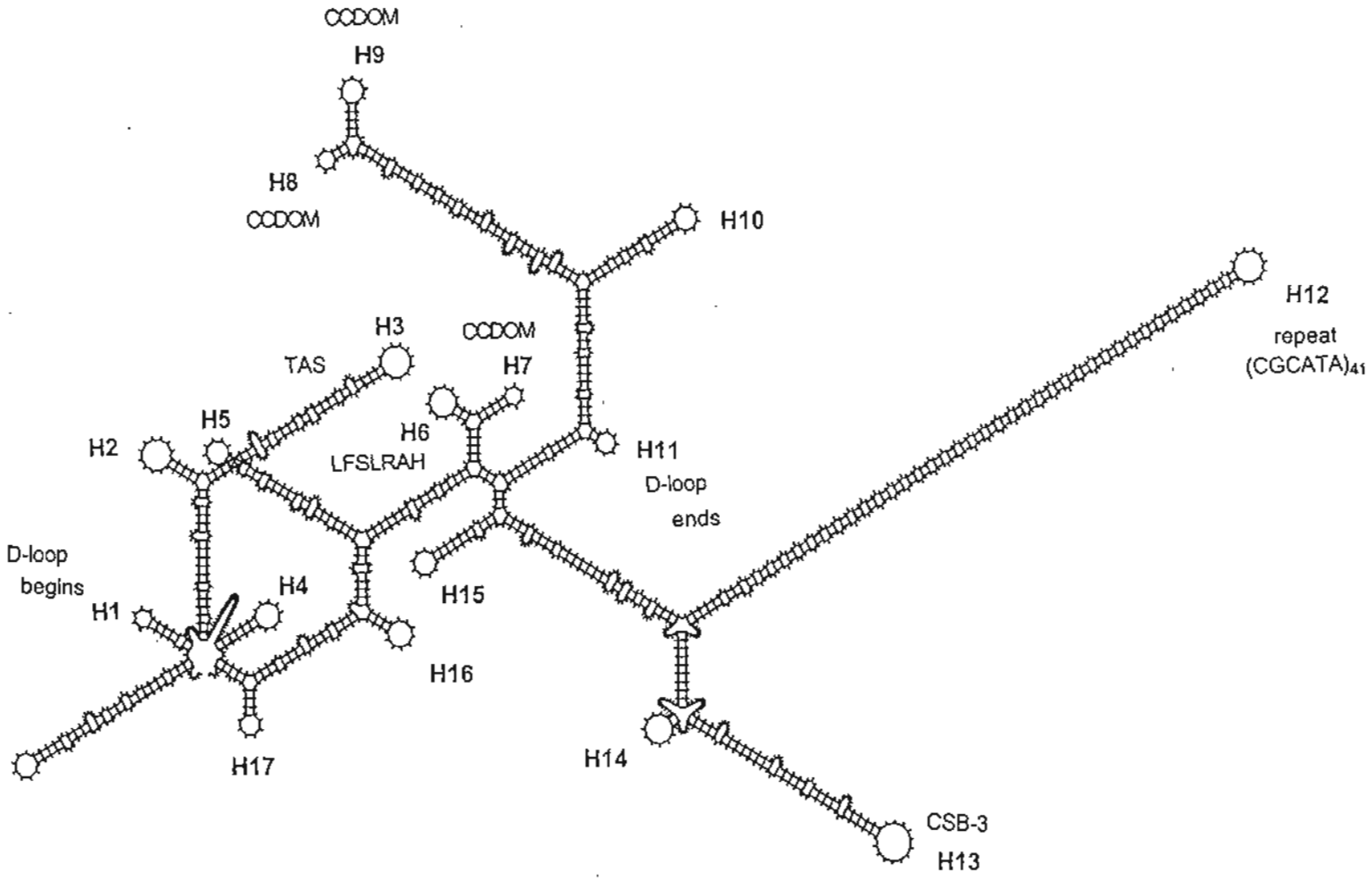

Figure 6.6: Secondary structure of the entire control region of mtDNA for a Torres Strait dugong (sample T677). H1 to H17 refer to 'hairpin' structures. This structure, consisting of 1490 bases of the control region, was composed by the program RNA 2° structure. The free energy of the structure was -284.8 kcal/mole. The sequence and placement of the hairpin structures are indicated in figure 6.2.

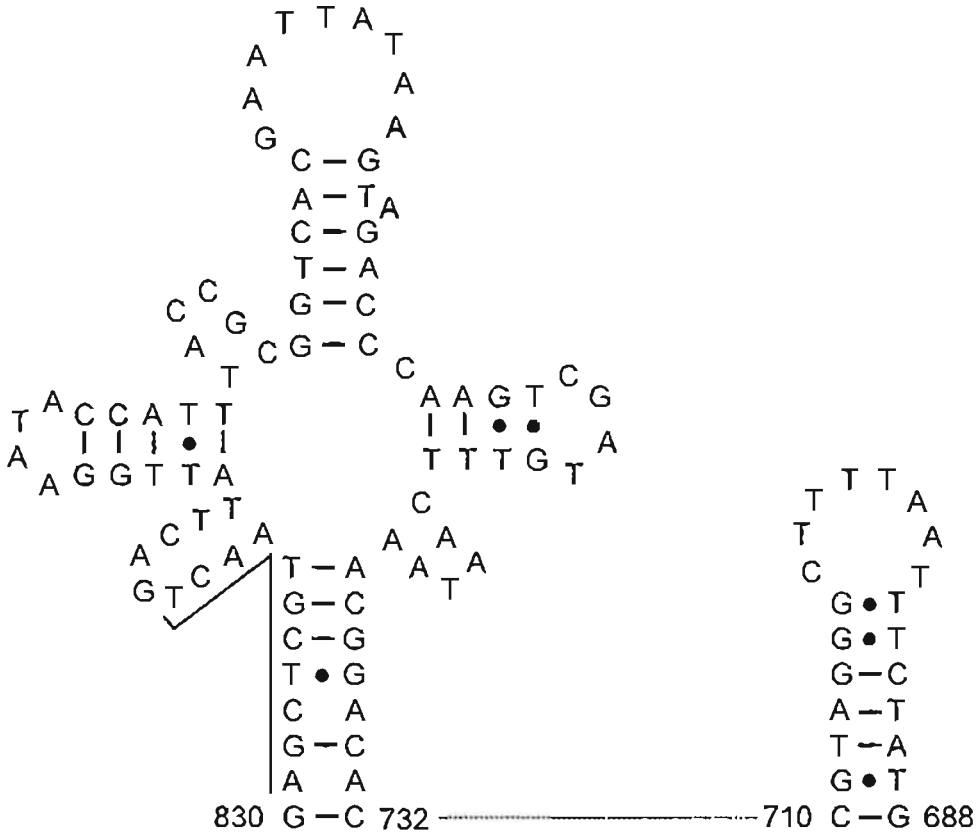

Figure 6.7: Secondary structure predicted for the CSB-1 segment of the control region. The structure was initially constructed using 15% suboptimality in MFOLD with a free energy of -4.5 kcal/mole, and was then modified to resemble the secondary structures predicted by Brown *et al.* (1986). The numbers at the base of the stems refer to the nucleotide numbers of the Torres Strait dugong (sample T677) in figure 6.2. Omitted sequence is indicated by the dotted line. The underlined sequence represents CSB-1 sequence. Dashes between bases indicate Watson-Crick pairing, and dots represent non canonical pairing.

6.4 DISCUSSION

6.4.1 Sequence characteristics

The lower occurrence of the base guanine (G) in the dugong control region (figure 6.3) suggests that nucleotide substitutions are not completely neutral, a trend which has been previously reported (Brown, 1985). The observed bias towards AT content in preference to GC (figure 6.3) has been recognised in other species. The underlying mechanism for differential occurrence of the four nucleotides is still undetermined and open to further study.

The transition to transversion ratio among the dugong sequences (further examined in Chapter 7) is extremely high. This ratio decreased (as expected) when the dugong sequence was compared to that of the Florida manatee (figure 6.2). This pattern is consistent in other regions of the mtDNA molecule (e.g., the cytochrome *b* gene, Chapter 5) and supports the pattern of transition to transversion ratios found among other species (Brown & Simpson, 1982; Brown *et al.*, 1982; Aquadro & Greenberg, 1983; Cann & Wilson, 1983; Thomas & Beckenbach, 1989).

6.4.2 Hypervariable region 1

The short region of the D-loop (194 bases) positioned between the Pro-tRNA gene and the central conserved block of the D-loop is probably a homologue of the 'hypervariable region 1' recognised in humans (Wakeley, 1993). The number of substitutions between the two dugong sequences from Torres Strait and Hervey Bay, was five to 15 fold higher in HVR-1, compared to the central conserved domain and right domain (i.e., HVR-2 (Wakeley, 1993)) respectively. In humans, the variability of the HVR-1 is only twice that of HVR-2 (Aquadro & Greenberg; 1983; Wakeley, 1993).

The variation observed for the control region as a whole (1.3%) is much higher than that (0.3%) among the dugong samples sequenced for the cytochrome *b* gene (Chapter 5). As highly variant regions of DNA sequence are expected to provide the level of information required for a population level study of the dugong, HVR-1 was selected in preference to the cytochrome *b* gene to provide genetic markers as detailed in Chapter 7.

6.4.3 Conserved regions

The dugong control region sequence has the major (conserved central domain) and minor (CSB-1, CSB-2 & CSB-3) conserved blocks common to most mammals (figures 6.4 & 6.5).

It was possible to align the dugong sequence to the highly conserved (seven codon) motif in the putative ORF region identified by Saccone *et al.* (1987), as well as to locate a number of putative start and stop sites (figure 6.2). Variation in start and stop sites of this putative ORF has been observed among a number of mammals (Saccone *et al.*, 1987; Hoelzel *et al.*, 1991). Consequently, there were length variations in the putative ORF region. There is no similarity at the amino acid level between the dugong and other mammals (Saccone *et al.*, 1987; Hoelzel *et al.*, 1991). In the elephant seal, the conserved seven codon motif is disrupted by a stop codon, supporting the suggestion that the putative ORF is not functional (Hoelzel *et al.*, 1993b). In the dugong, the putative ORF is disrupted by a stop codon 30 bases upstream from the conserved seven codon motif (figure 6.2). Although relatively conserved among mammals (with very high conservation in the seven codon segment), the putative ORF region does not reflect the pattern of strict conservation expected in functional elements (Hoelzel *et al.*, 1991).

Because of the high sequence conservation between species (particularly the seven 'codon' motif), the putative ORF suggests evolutionary constraint, and may be the remains of a previously functional gene (Zyskind *et al.*, 1983; Hoelzel *et al.*, 1991). Another possibility is that the putative ORF is not under evolutionary constraint at the nucleotide or amino acid level, rather it may be conserved to support a secondary structure feature. It is interesting that one of the hairpin structures (H6, within a cloverleaf) in the folded dugong sequence coincides with the conserved codon sequence of the putative ORF region (figure 6.6; section 6.4.5).

6.4.4 Repetitive region

Regions of tandem repeats are dispersed throughout the control region (see table 6.2), except in the blocks of sequence that are recognised to be highly conserved among mammals (i.e., CCDOM, CSB-1, CSB-2, CSB-3, CSB2+3, TAS). The location of the repeat motifs can be taxon specific. However, the number of copies and the repeat motif sequences do not appear to be

Table 6.2: Repeat DNA present in the mtDNA control region of various vertebrates. These repeats are often heteroplasmic. LD and RD refer to the left and right domain of the control region respectively, as schematically illustrated in figure 6.1. The precise location of the repeat in either the left and right domain are only given if indicated by the authors. bp and kb refer to base pairs and kilo base pairs respectively. This table has been adapted from Hoelzel *et al.* (1994) and Fumagalli *et al.* (1996).

Organism	*Species*	*Locality as in figure 6.1*	*Repeat motif*	*Reference*
Fish	*Alosa sapidissima*	control region	1.5 kb	Bentzen *et al.* (1988)
	Acipenser transmontanus	LD	82 bp	Buroker *et al.* (1990); Brown *et al.* (1992)
	Gadus morhua	LD	40 bp	Johansen *et al.* (1990); Arnason & Rand (1992)
	Cyprinella spiloptera	RD[c]	260 bp	Broughton & Dowling (1994)
Amphibians	*Xenopus laevis*	LD	45 bp	Dunon-Bluteau & Brun (1987)
	Rana catesbeiana	LD	40 bp	Yoneyama (1987)
	Triturus sp.	control region	140 bp; 1.1 kb	Wallis (1987)
	Rhacophorus taipeianus	LD[a]	40 bp	Yang *et al.* (1994)
Reptiles	*Cnemidophorus* sp.	control region	64 bp; 0.8-9.0 kb	Densmore *et al.* (1987); Moritz & Brown (1987)
	Heterontoia binoei	control region	≤10.4 kb	Moritz (1991)
Birds	*Rallus* sp.	control region	180 bp	Avise & Zink (1988)
	Pomatosomus temporalis	control region	200 bp	Edwards & Wilson (1990)
Mammals	*Nycticeius humeralis*	LD	81 bp	Wilkinson & Chapman (1991)
	Sorex sp.; *Crocidura* sp.	LD & RD[b]	78-80 bp & 12-14 bp[b]	Stewart & Baker (1994a; 1994b); Fumagalli *et al.* (1996)
	Oryctolagus cuniculus; Lepus capensis; Sylvilagus floridanus	RD	20 bp[b]; 153 bp[c]	Mignotte *et al.* (1990); Biju-Duval *et al.* (1991); Casane *et al.* (1994)
	Ornithorhynchus anatinus	RD[b]	9-12 bp & 17 bp	Gemmell *et al.* (1996)
	Cercopithecus aethiopus	RD[ac]	108 bp	Karawya & Martin (1987)
	Macaca fuscata	RD[ac]	158-168 bp	Hayasaka *et al.* (1991)
	18 carnivore sp.	RD[ab]	6-30 bp	Hoelzel *et al.* (1994)
	Halichoerus grypus	RD[b]	6 bp; 14 bp	Arnason *et al.* (1993b)
	Mirounga sp.	RD[b]	20 bp; 38 bp	Hoelzel *et al.* (1993b)
	Phoca vitulina	RD[b]	6 bp; 8 bp	Arnason & Johnsson (1992)
	Equus caballus	RD[b]	8 bp	Xu & Arnason (1994)
	Diceros bicornis	RD[ab]	10 bp	Jama *et al.* (1993)
	Sus scrofa	RD[ab]	10 bp	Ghivizzani *et al.* (1993)
	Dugong dugon	RD[b]	6 bp	this study

[a] The entire mtDNA control region has not been sequenced.
[b] The repeats are positioned between CSB-1 and CSB-2.
[c] The repeats are positioned between CSB-2 and the Phe-tRNA gene.

conserved within a taxon (table 6.2). Furthermore, the occurrence of repeats in the control region is not universal.

The position of repeats of DNA at the 3' end of the dugong control region (figure 6.1), is consistent with most other mammals (table 6.2). Of the mammalian species in table 6.2, the dugong repeats are the most similar in type to repeats found in the pig (Ghivizzani *et al.*, 1993). Both are located in a similar region of the control region (i.e., between CSB-1 and CSB-2), and both are lengthy tandem alternating purine-pyrimidine sequences. Ghivizzani *et al.* (1993) reviewed and discussed the functional implications of repeating sequences, including the ability of perfectly alternating purine-pyrimidine sequences within a transcriptionally active region of the D-loop sequence to convert to left handed Z-DNA, a conformation which may have functional significance *in vivo*.

The repetitive DNA found in the control region of dugong mtDNA not only varies in length between individuals, but also appears to exhibit heteroplasmy as indicated by the two clones sequenced for a Moreton Bay dugong (sample D3). The heteroplasmy was evident in the intervening sequence which disrupts the repeat (section 6.3.3). Length heteroplasmy is not as rare in mtDNA as previously believed (Buroker *et al.*, 1990; Ghivizzani *et al.*, 1993; Casane *et al.*, 1994; Hoelzel *et al.*, 1994; Xu & Arnason, 1994; Bendall & Sykes, 1995), particularly with respect to repetitive DNA of the control region (table 6.2). Because of the individual variation in length, this repeat was further investigated for utility in recognising intraspecific subdivision for the dugong (Chapter 9).

6.4.5 Secondary structure

Presumably, constraints to variation are a consequence of functional requirement (Wakeley, 1993). Although the control region is a rapidly evolving and non coding segment of mtDNA, variation in parts of this region indicate constraint. The consistent occurrence of repeat regions among taxa, specifically between the conserved blocks of sequence, provides further evidence towards a significant function for the control region. As the control region is non coding, it has been speculated that secondary structure is the level at which the evolutionary pressure is acting. As a consequence, a number of researchers (e.g., Brown *et al.*, 1986; Hoelzel *et al.*,

1991; Saccone *et al.*, 1991; Gemmell *et al.*, 1996) have proposed secondary structures that are conserved among taxa.

Similarly, I was able to locate secondary structures, for both the entire control region (figure 6.6) and one of the structures predicted by Brown *et al.* (1986) (figure 6.7). However, the secondary structure of the shorter control region sequence (figure 6.7) had to be adapted so that it resembled the predictions of Brown *et al.* (1986), and I was therefore sceptical of its validity. It is likely that the secondary structure of DNA in a cell is affected by pH, temperature, or mineral content. Programs that construct secondary structures do not incorporate such factors, and the structures are therefore putative. The following is an attempt to discern some pattern from the secondary structure of the entire control region (figure 6.6).

The structure in figure 6.6 is a representative of a multitude of similar structures which altered depending on the amount of sequence information provided. Figure 6.6 illustrates a number of hairpin structures located throughout the control region, which were often associated with conserved segments such as, the seven codon motif in the putative ORF, the CSB-3, tRNA-Pro and tRNA-Phe. However, these hairpin structures were not exclusively associated with conserved sequence, as exemplified by the particularly large hairpin associated with the repeat region, and multiple hairpins (comprising a complex of cloverleaf structures) in HVR-1 (figure 6.6). Brown *et al.* (1986) also found the HVR-1 sequence was able to fold into cloverleaf structures in various vertebrates (i.e., TAS elements), and believed these to be of functional significance. The position of hairpin structures in the entire dugong control region (figure 6.6) did not strictly conform with the formation of cloverleaf structures that have been associated with the CSB–1, TAS elements, and the 5' and 3' ends of the control region of various vertebrates (Doda *et al.*, 1981; Walberg & Clayton, 1981; Brown *et al.*, 1986).

6.5 CONCLUSION

The dugong control region sequence exhibits a number of characteristics common to other vertebrates/mammalian species. For this reason, any general explanations from these other species regarding control region functions may be confidently extended to include the dugong.

Compared to the 0.3% observed for the cytochrome *b* gene, the Moreton Bay and Torres Strait dugongs had a sequence difference of 1.6% in the control region, of which 84% was located in the 194 bases of HVR-1 (5' of the central conserved domain). Because of its high diversity, and because 194 bases could be easily sequenced in a single run, HVR-1 was chosen to examine intraspecific genetic structure of the dugong as detailed in Chapter 7. The microsatellite repeat (3' of the central conserved domain) was also assessed as intraspecific genetic marker in Chapter 8, as it was expected to be highly variable among dugongs.

CHAPTER SEVEN

DUGONG GENETIC STRUCTURE INFERRED FROM THE HYPERVARIABLE REGION 1 (CONTROL REGION) OF mtDNA

This chapter details the genetic structure of the dugong using the hypervariable region 1 (HVR-1) of the control region of mtDNA (defined in Chapter 6). A total of 194 bases of HVR-1 were amplified and sequenced from 103 dugongs and one Florida manatee (as indicated in Chapter 6). Only Asian haplotypes (n=7) formed a cluster on the gene trees which was geographically restricted from dugong populations elsewhere. Two mtDNA haplotype clusters were evident for Australian dugongs, one cluster predominately represented by samples from northwest Australia, the other mainly represented by samples from southeast Australia. These clusters overlapped broadly in the Great Barrier Reef region. Analyses of haplotypes within each cluster separately, indicated genetic substructure along the east Queensland coast.

7.1 ANALYTICAL METHODS

Intraspecific units were initially nominated primarily on the basis of geography, and with some consideration of the clustering of haplotypes in phylogenetic trees constructed by PAUP and PHYLIP (sections 7.1.1 & 7.1.2 respectively). Using AMOVA (section 7.1.3), MacClade (section 7.1.4) and REAP (section 7.1.5), gene flow among the nominated intraspecific units was investigated. REAP was used to estimate N_{ST} values which in turn, were used to estimate female migration rates (Chapter 8).

7.1.1 PAUP

PAUP (Macintosh, Version 3.1.1, Swofford, 1993) calculated the most parsimonious tree from aligned haplotypes. Characters were defined as

unordered, such that any state (i.e., base/nucleotide) was capable of transforming directly to any other state, with equal cost.

A heuristic search (because the data set was too large to permit the use of exhaustive methods) was used to construct a gene tree using the nearest neighbour interchange (NNI) branch swapping method. Branch swapping is a technique which swaps neighbouring branches of the previous arrangement on a gene tree, and has a better chance of finding the optimum tree compared to the stepwise-addition method (another tree finding option available in PAUP). Branches having a maximum length of zero were collapsed to yield polytomies. No topological constraints were enforced and the gene tree was polarised by including the sample from the Florida manatee as an outgroup.

Felsenstein's (1985) bootstrap approach was used to place confidence estimates on haplotype clusters found in the most parsimonious trees obtained via PAUP. Numbers provided on the nodes of the consensus tree indicate the percentage of the bootstrap replications that found the haplotype cluster descending from that branch. As my data contained almost as many informative (polymorphic) sites as there are haplotypes, and because haplotypes were closely related (often differing by only one or two bases), bootstrap values >95% were expected to be rare. Percentages greater than 80 are considered as 'good support', with some indication of reliability provided by a bootstrap value of 50 to 80% (see Hillis *et al.* (1996) for a detailed explanation on this).

7.1.2 MEGA and PHYLIP

A distance matrix from pairwise comparisons of haplotypes was constructed by applying the Tamura-Nei algorithm (Tamura & Nei, 1993, see Chapter 8) using the program MEGA (Molecular Evolutionary Genetics Analysis, IBM compatible, Version 1.0, Kumar *et al.*, 1993). From this distance matrix, a tree was created using the Neighbour Joining method in PHYLIP (Macintosh, Version 3.5c, Felsenstein, 1989). The Neighbour Joining method infers an additive tree and does not assume ultrametric data (i.e., does not assume that all lineages have diverged by equal amounts) (Swofford & Olsen, 1990). The Florida manatee sample was included in the Neighbour Joining tree to act as an outgroup.

7.1.3 AMOVA

AMOVA (Analysis of Molecular Variance, IBM compatible, Version 1.55, Excoffier *et al.*, 1992) was used to quantify the geographic differentiation of the mtDNA haplotypes by producing estimates of variance components and ϕ-statistics. ϕ-statistics are haplotype statistics comparable to F_{ST} of Weir & Cockerham (1984), and are calculated by using both haplotype frequency and sequence divergence (Hudson *et al.*, 1992).

F-statistics were introduced by Wright (1931, 1943, 1951) to measure the partitioning of genetic variance among populations. According to Wright (1931; 1943; 1951) and the analogous measures of Weir & Cockerham (1984), there are three basic statistical quantities when diploid individuals are sampled from a series of populations. The overall inbreeding coefficient (or fixation index) F_{IT} is the correlation of genes within individuals; F_{ST} is the correlation of genes of different individuals in the same populations; and F_{IS} is the correlation of genes within individuals within populations (Hillis & Moritz, 1990; Avise, 1994). Their interrelationship is as follows:

$$(1 - F_{IT}) = (1 - F_{ST})\,(1 - F_{IS})$$

Populations, although dependent through their shared ancestry, are assumed to have been isolated since the coancestral population, and are randomly sampled. Therefore individuals and their genes in one population are assumed to be independent of those in another (Hillis & Moritz, 1990; Avise, 1994).

In AMOVA, the significance of the variance components and ϕ-statistics are tested using a permutation approach, eliminating the normality assumption that is conventional for analysis of variance but inappropriate for molecular data (Excoffier *et al.*, 1992). ϕ_{ST} is the correlation of random haplotypes within populations, relative to that of random pairs of haplotypes drawn from the whole species; ϕ_{CT} is the correlation of random haplotypes within a group of populations, relative to that of random pairs of haplotypes drawn from the whole species; and ϕ_{SC} is the correlation of the molecular diversity of random haplotypes within populations, relative to that of random pairs of haplotypes drawn from a group of populations (Excoffier *et al.*, 1992). Therefore, there are three genetic variance

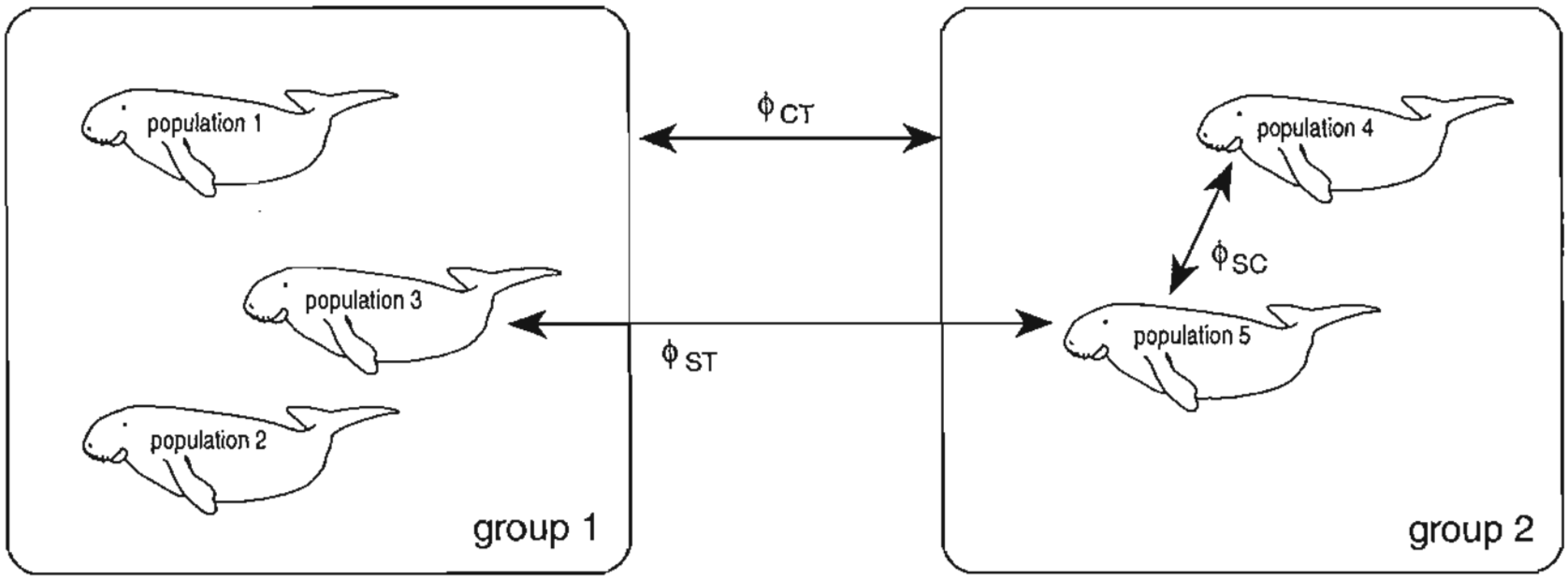

Figure 7.1: AMOVA hierarchical analysis. The three genetic variance components calculated by AMOVA (Excoffier *et al.*, 1992) are among groups (ϕ_{CT}), among populations within groups (ϕ_{SC}) and among populations (ϕ_{ST}).

components calculated by AMOVA: within populations (ϕ_{ST}), among groups (ϕ_{CT}), among populations within groups (ϕ_{SC}).

This version of a nested analysis is illustrated in figure 7.1. In this study, the term 'geographic unit' equates to that of 'group' used by Excoffier *et al.*, (1992). Dugong groups were nominated on the basis of their geography and/or the genetic type of each population, and the significance of the groupings tested via a series of AMOVA tests. Dugong samples collected beyond the dugong's recognised range on the south east coast of Australia (i.e., New South Wales), were assumed to have originated from the Hervey Bay dugong population for two reasons: 1) because of their genetic type, and 2) the coincidence of the timing of sample collection with the mass exodus of dugongs from Hervey Bay after an extreme weather event (Preen & Marsh, 1995, see also the discussion on this in section 7.3.2.2).

The form of the input data to AMOVA was a list of groups, the populations contained therein, and the frequency of each haplotype in each population. In addition, a distance matrix of pairwise comparisons of haplotypes was provided.

7.1.4 MacClade

Slatkin & Maddison (1989) suggest using the number of migration events required by a tree as a statistic to measure gene flow. I implemented this test using the program MacClade (Macintosh, Version 3, Maddison & Maddison, 1992). A consensus tree from PAUP was imported and a MacClade data file was constructed listing all of the specimens, treating groups as an unordered character.

The groups were then traced for the minimum number of inter-group migration steps allowed by the tree. After omitting the outgroup, a chart was created showing the number of inter-group migration steps from 1000 random joining/splitting trees. The distribution of these should not include the observed number of steps inferred from the gene tree if a restriction to gene flow exists (Maddison & Maddison, 1992; Slatkin & Maddison, 1989).

A second file was constructed in MacClade containing only one example of each haplotype and the Florida manatee sequence in order to obtain interspecific and intraspecific sequence statistics.

7.1.5 REAP

MONTE and DA are two programs of the nine program package, REAP (Restriction Enzyme Analysis Package, IBM compatible, Version 4.0, McElroy *et al.*, 1992). MONTE investigates the extent of geographic heterogeneity among haplotype frequency distributions through a Monte Carlo simulation (Roff & Bentzen, 1989). This procedure is designed to minimise the effect of large numbers of empty cells on the validity of chi-square analysis. The extent of heterogeneity in the original matrix is compared to that estimated from repeated randomisations of the data. The output file reports: 1) the probability (including standard errors) of generating the observed χ^2 value by chance alone, which exceeds that calculated from the original matrix, and 2) average, minimum and maximum χ^2 values from the simulation, as well as a cumulative frequency distribution of χ^2 values.

A corrected distance matrix (Appendix F) added to the input file for MONTE, created the input for the program DA. DA handles DNA sequence data to estimate haplotype and nucleotide diversity within geographic units, and computed nucleotide diversity and divergence among geographic units. Haplotype diversity was estimated according to Nei (1987, eqs. 8.4, 8.5 and 8.12). Nucleotide diversity and nucleotide divergence were estimated according to Nei & Tajima (1981) and Nei (1987, eqs. 10.7, 10.19, 10.20 and 10.21).

7.1.6 Nucleotide Diversity Analysis (NDA)

The problem with nested analysis of variance (e.g., AMOVA, Excoffier *et al.*, 1992), is that the hierarchical structure of the data must be inferred before beginning the analysis. Holsinger & Mason-Gamer (1996) present a method that partitions diversity into hierarchical components allowing any structure present in the data to emerge naturally. Based on Nei's (1982) nucleotide diversity statistics with a correction for bias, Holsinger & Mason-Gamer's (1996) statistic provides a direct analog to Wright's (1951; 1965) F_{ST}, appropriate for haplotype sequence data. The Nucleotide Diversity Analysis

(NDA) input required aligned haplotypes, and their observed frequency in each population.

7.2 RESULTS

From 103 dugongs sampled from 24 populations (figure 3.2), 37 haplotypes with 39 variable sites (not including the outgroup) were identified (table 7.1). Haplotype #15 was the most frequently occurring haplotype and was observed in 24% of the dugongs sampled (figure 7.2). Of the 37 haplotypes, 24 were individual-specific (figure 7.2). However, it was common for haplotypes to be shared between neighbouring populations along the Australian coast (see table 7.2 & figure 3.2). Of the four dugong specimens from the West Indian Ocean, one had a haplotype common among East Australian dugongs (haplotype #15), whereas the other three had a haplotype unique to the West Indian Ocean (haplotype #35). None of the five Asian haplotypes (n=7) were shared with Australian or West Indian Ocean dugong populations. The Australian sample (n=92) had 30 Australian-specific haplotypes.

Sequence statistics prepared using MacClade (section 7.1.4) indicate an unequal expression of the bases among the dugong HVR-1 sequences, with a lower occurrence of the base guanine (G). Similar frequencies were obtained when the Florida manatee HVR-1 sequence was included: A, 28.4%; C, 28.7%; G, 13.4%; T, 29.5%. Such a bias against guanine, was similarly observed for the entire control region (figure 6.3). Of all the 39 base differences among the dugong haplotypes, only one was a (TA) transversion (the others being transitions: 69.1% CT; 30.9% GA). An uneven distribution of the occurrence of polymorphic sites within HVR-1 was suggested by a 'variable site histogram' (constructed in MacClade, data not shown) and further investigated in Chapter 8.

7.2.1 PAUP

The total number of gene tree rearrangements (including the outgroup) tried by PAUP was 1584, with the length of the shortest trees found being 121 steps and a total of four equally parsimonious trees being retained. They differed only in slight re-arrangements among the terminal branches. The consensus of these four trees did not vary according to the method used for constructing it (i.e., Strict, Semistrict, Adams, Majority Rule).

Table 7.1: Aligned mtDNA haplotypes. Next to the sequences are (arbitrary) haplotype numbers, and the geographic units in which each haplotype was found: *A* (Asian), *F* (West Indian Ocean), *N* (North & West Australian), *G* (Great Barrier Reef), and *S* (South East Queensland). Numbers in the first row indicate polymorphic sites in the 194 bases of the hypervariable region 1 (these numbers varied when comparing dugong sequences to the outgroup because of insertions/deletions). Haplotype #2 is the reference sequence. A period (.) indicates common sequence, with variable sites indicated by the base concerned. The outgroup reserves haplotype #1, but was not included (see figure 6.2 for Florida manatee sequence).

	POLYMORPHIC SITES IN HYPERVARIABLE REGION 1																																						
HAPLOTYPE	2	5	8	24	25	26	27	29	33	34	35	36	38	39	42	43	45	48	51	53	55	56	60	61	90	91	94	98	107	113	116	122	150	153	154	170	178	179	187
2 N	C	T	G	T	A	A	A	C	A	G	T	A	T	T	T	C	C	T	T	C	A	C	T	A	T	T	T	C	T	C	C	A	T	C	G	C	G	A	T
3 N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.
4 N	.	.	A	.	.	.	.	.	.	.	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	C	T	.	.	.	.	.
5 N	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6 N,G,S	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	C	T	.	.	.	G	.
7 N	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
8 N	.	.	A	.	.	.	.	.	.	.	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	C	T	.	.	.	G	.
9 N,G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.
10 N,G	.	.	A	.	.	.	.	.	G	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	.	C	T	.	.	.	G	.
11 G,S	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.
12 G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
13 G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.
14 N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.
15 G,S,F	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	T	.	C	T	.	.	.	G	.
16 G	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	C	.	.	.	C	.	C	.	.	.	.	.	T	.	C	T	.	.	.	G	.
17 N	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.
18 S	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	T	.	C	T	.	.	.	.	.
19 S	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	.	C	.	.	.	.	T	.	C	T	.	.	.	G	.
20 N,G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
21 A	T	.	A	C	.	.	.	.	.	A	.	G	.	C	C	.	.	.	.	.	.	.	C	G	C	.	.	.	.	T	.	G	.	.	.	.	.	G	.
22 A	T	.	A	C	.	G	.	.	.	A	C	G	.	C	C	.	T	C	.	.	.	.	C	.	C	.	.	.	.	T	.	G	.	.	.	.	.	G	.
23 N	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
24 S	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	.	C	.	.	.	.	T	.	C	T	.	.	.	G	C
25 S	.	.	A	.	.	G	.	.	G	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	G	C	T	.	.	.	G	.
26 G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	T	.	.	.	.	.
27 A	T	.	A	C	.	.	.	.	.	A	C	G	.	C	C	.	T	C	.	.	.	.	C	G	C	.	.	.	.	T	.	G	.	.	.	.	.	G	.
28 A	.	.	A	.	.	.	.	.	.	A	.	.	C	.	C	.	T	C	.	T	.	.	C	.	C	.	.	T	.	T	.	.	.	.	A	.	A	G	.
29 G	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	C	.	.	.	C	T	.	.	.	G	.
30 G	.	.	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	C	.	.	.	.	.	T	.	.	.	.	.	.	G	.
31 G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	G	.	.	.	T	.	.	.
32 G	.	C	A	.	.	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	.	C	.	.	.	.	T	.	C	T	.	.	.	G	.
33 G	.	.	A	.	G	.	.	.	.	A	.	.	C	C	C	T	.	C	.	.	.	.	C	.	.	C	.	.	.	.	T	.	C	T	.	.	.	G	.
34 N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
35 F	.	.	A	.	.	.	.	.	.	A	C	.	C	.	C	T	.	.	.	.	G	T	C	.	C	.	.	.	.	.	.	G	.	T	.	.	.	G	.
36 G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	A	.	.	.	.	G	.	.	.	.	.	.	.
37 N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.
38 A	T	.	A	C	.	.	.	.	.	A	C	.	C	C	C	.	.	C	.	.	.	.	C	.	C	.	.	.	.	.	.	G	.	.	.	.	.	G	.

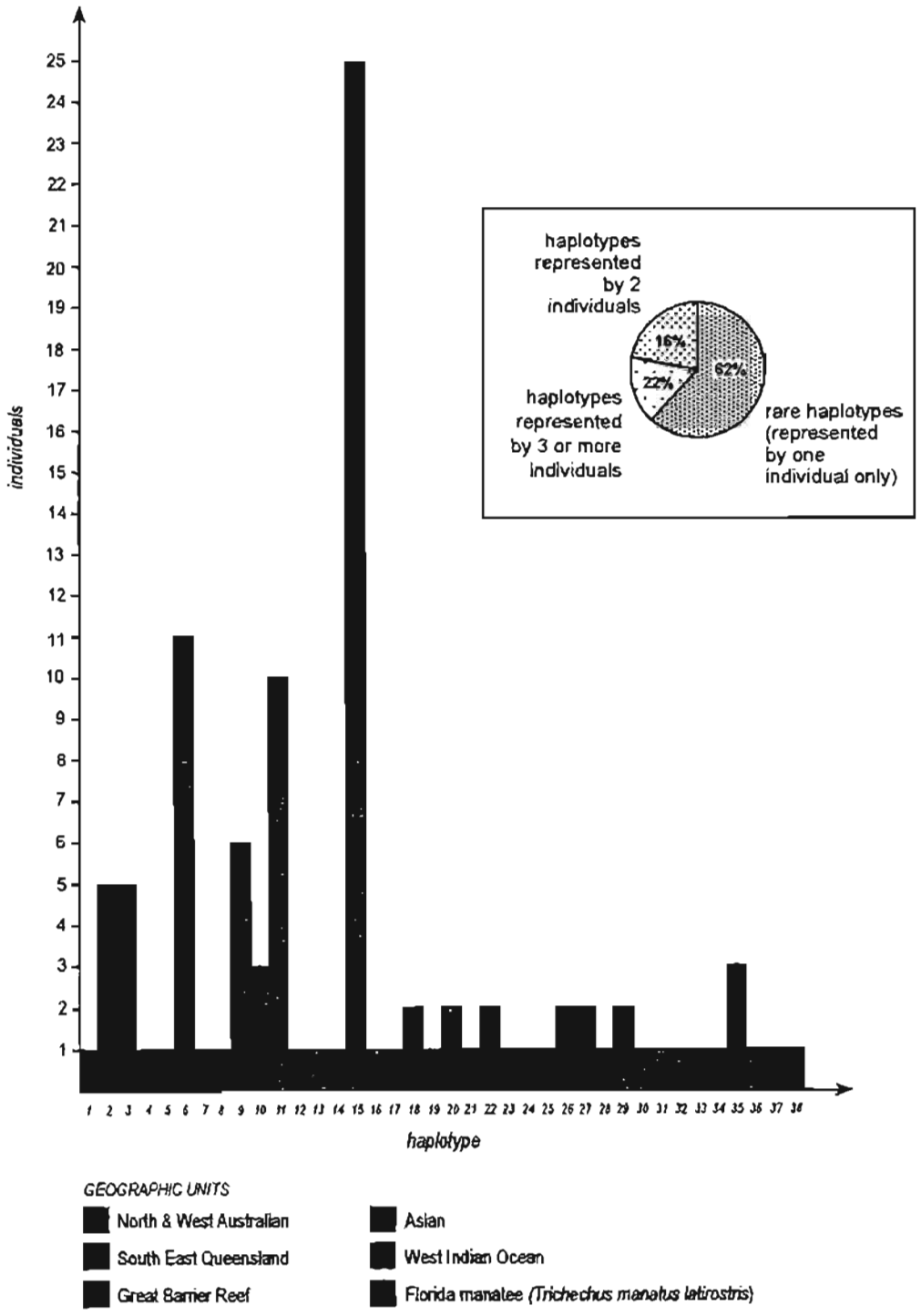

Figure 7.2: Haplotypes and their frequencies among individuals. Number of dugongs (and manatee) representing each haplotype and the geographic units to which they have been nominated. The distribution of haplotypes and their frequencies are also detailed in figure 7.5 and table 7.2 respectively.

Table 7.2: Samples included in the analysis. Each specimen is listed with its reference label, latitude (lat) and longitude (long) of the site of collection (population), number of individuals representing each population (ind), and haplotype numbers (H). The outgroup (Florida manatee) reserves haplotype #1 and is not included in this table.

Pop #	Population	LAT	LONG	Ind	Specimen	H
1	Taytay	10.47N	119.32E	2	Lem1	21
					Lem2	38
2	Ambon	3.41S	128.10E	2	MIK	22
					A4	22
3	Ranong	9.58N	98.35E	1	A1	28
4	Surat Thani	9.09N	99.20E	2	A2	27
					A3	27
5	Arabian Gulf	26.00N	52.00E	3	V3	35
					V78	35
					V11	15
6	Kenya	1.00N	38.00E	1	V12	35
7	Exmouth Bay	22.20S	114.09E	2	WA	17
					RIK	14
8	Broome	17.58S	122.15E	4	BM1	20
					BBO	3
					BB1	34
					BB2	3
9	Darwin	12.23S	130.44E	1	Darwin	2
10	Borroloola	16.00S	136.15E	1	SmB	37
11	Torres Strait	10.23S	142.02E	21	T673	9
					T674	6
					T675	6
					T676	2
					T677	2
					T678	2
					T679	10
					TS1	2
					TS2	3
					TS3	23
					TS4	4
					TS5	3
					TS6	5
					TS7	6
					TS8	6
					TS9	7
					TS10	8
					TS43	6
					TS44	6
					TS45	3
					Friday	10
12	Lockhart River	12.58S	143.31E	3	LH1	11
					LH2	10
					LH8	13
13	Hopevale	35.48S	142.14E	4	HV1	11
					HV2	11
					HV3	6
					HV4	11
14	Cairns	16.51S	145.43E	3	GK	15
					Cairns(319)	16
					Yarra	11

Pop #	Population	LAT	LONG	ind	Specimen	H
15	Townsville	19.16S	146.49E	9	313	9
					315	9
					317	15
					321	15
					322	11
					323	9
					327	26
					328	15
					329	9
16	Bowen	20.00S	148.10E	1	Bowen	12
17	Shoalwater Bay	22.22S	150.23E	15	SB1	11
					SB2	29
					SB3	26
					SB4	30
					SB5	31
					SB6	6
					SB7	15
					SB8	15
					SB9	30
					SB10	32
					SB11	33
					SB12	15
					SB13	15
					SB14	36
					SB15	11
18	Rockhampton	23.22S	150.32E	2	King	9
					Rocky	20
19	Hervey Bay	24.67S	152.40E	7	HB1	24
					HB2	15
					HB3	19
					HB4	15
					HB5	11
					HB02	15
					Heart	15
20	Moreton Bay	27.26S	153.20E	12	CL1	15
					CL2	6
					CL3	6
					CL6	15
					CL7	15
					D1	6
					D2	15
					D3	18
					D4	16
					MB6	15
					314	15
					Head(320)	15
21	Iluka	29.25S	153.21E	1	PGU	15
22	Newcastle	32.55S	151.46E	3	ORC120	15
					ORC121	15
					PGB	25
23	Sydney	33.55S	151.10E	2	ORC107	18
					ORC139	11
24	Jervis Bay	35.03S	150.44E	1	ORC106	15

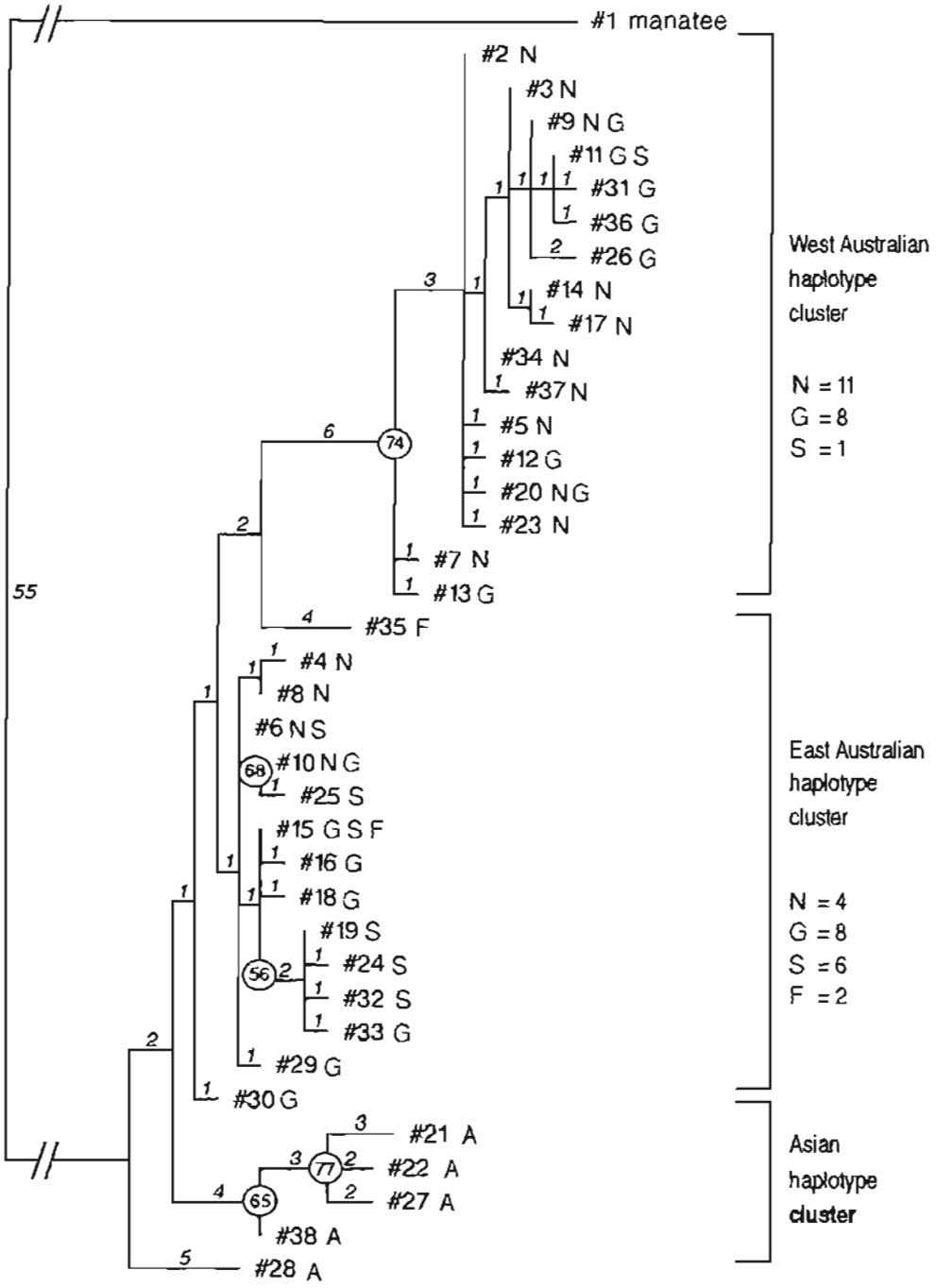

Figure 7.3: One of the four equally parsimonious (unrooted) trees found by PAUP. Three main haplotype clusters are evident: 1) West Australian, 2) East Australian, and 3) Asian. The two Australian haplotype clusters overlap in the region of the Great Barrier Reef. The total number of haplotypes from each geographic region are shown next to the bracketed clusters. Numbers at the branch terminals preceded with a #, are haplotypes as indicated in table 7.1. Next to the haplotypes are the geographic origins of that particular haplotype: N, North & West Australian; G, Great Barrier Reef; S, South East Queensland; A, Asian; F, West Indian Ocean. The number of state changes between haplotypes are indicated on the branches (in italics). Bootstrap values indicated within circles at the nodes, were produced by a 50% majority-rule consensus from 100 replciates. Consistency index = 0.702, Homoplasy index = 0.298, and the Retention index = 0.841.

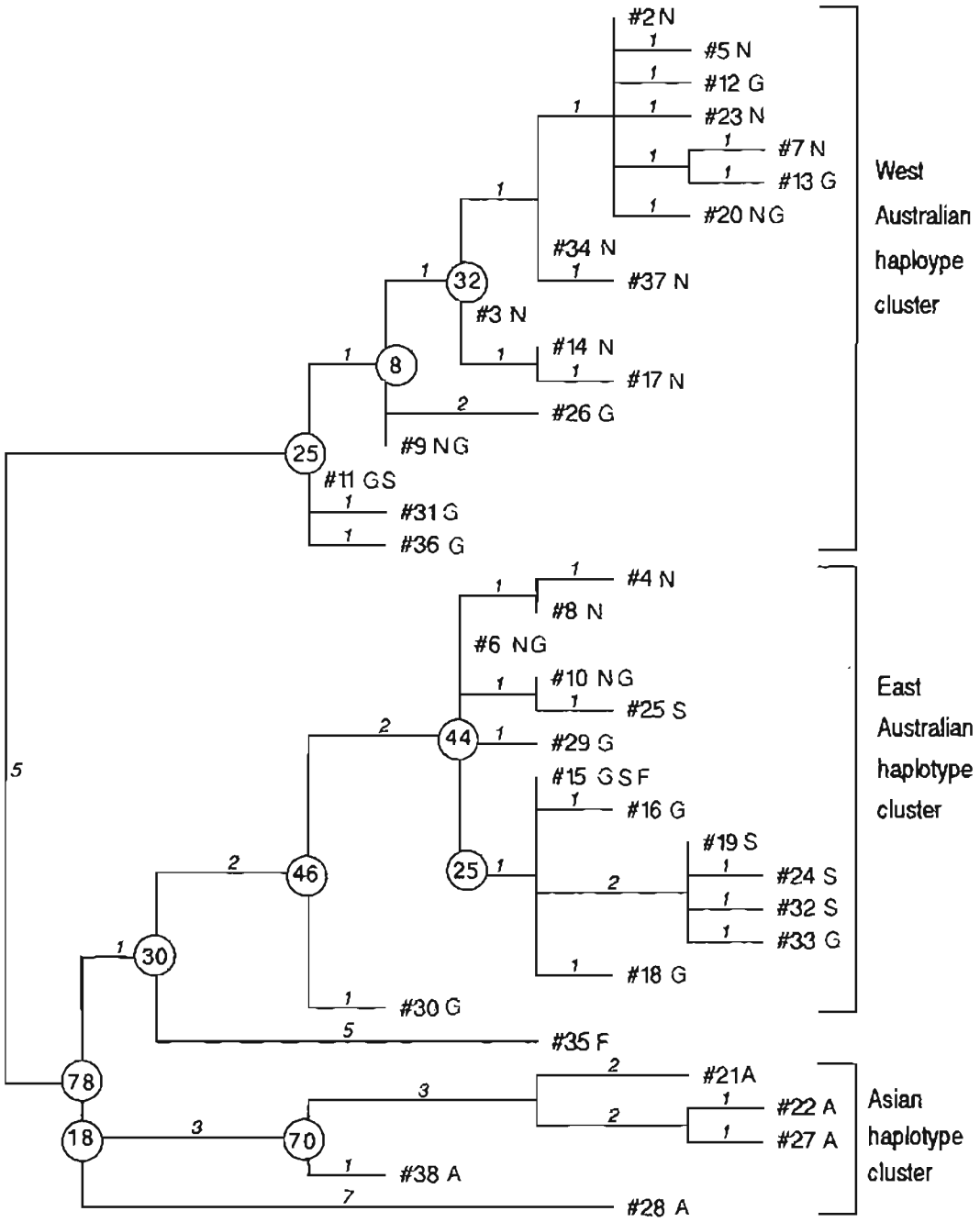

Figure 7.4: One of the 41 equally parsimonious trees found by PAUP (excluding the outgroup), with midpoint rooting. The outgroup has been omitted to emphasise the three haplotype clusters: 1) West Australian, 2) East Australian, and 3) Asian. Numbers at the branch terminals preceded by a #, are haplotypes as detailed in table 7.1. Next to the haplotype numbers are the nominated geographic units which are abbreviated as follows: N, North & West Australian; G, Great Barrier Reef; S, South East Queensland; F, West Indian Ocean; A, Asian. Distance between haplotypes are indicated by the number of state (nucleotide) changes, shown on the branches in italics. Values within circles at the nodes were obtained from a bootstrap of the data with 100 replicates, and are indicated for the deeper nodes only. Consistency index = 0.591. Homoplasy index = 0.409, and Retention index = 0.875.

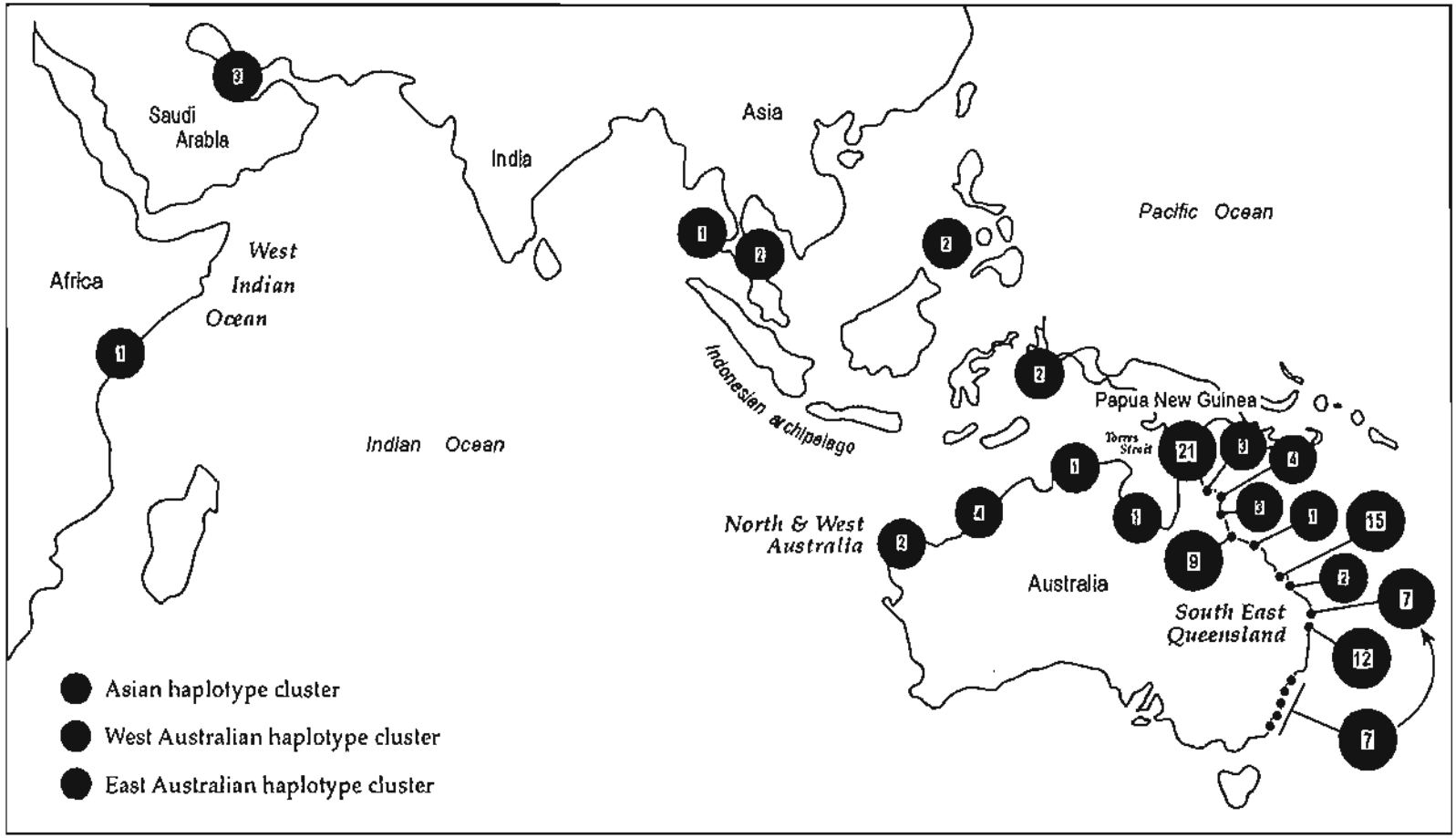

Figure 7.5: Geographic distribution of the haplotype clusters. The proportion of each haplotype cluster (shown in figure 7.4) represented in each sampled dugong population. The dugong populations which were sampled are listed in table 7.2. Sample sizes (n) of each population are indicated within each pie. Dugongs sampled from the New South Wales coast (Australia) are believed to belong to the Hervey Bay dugong population as indicated by the arrow.

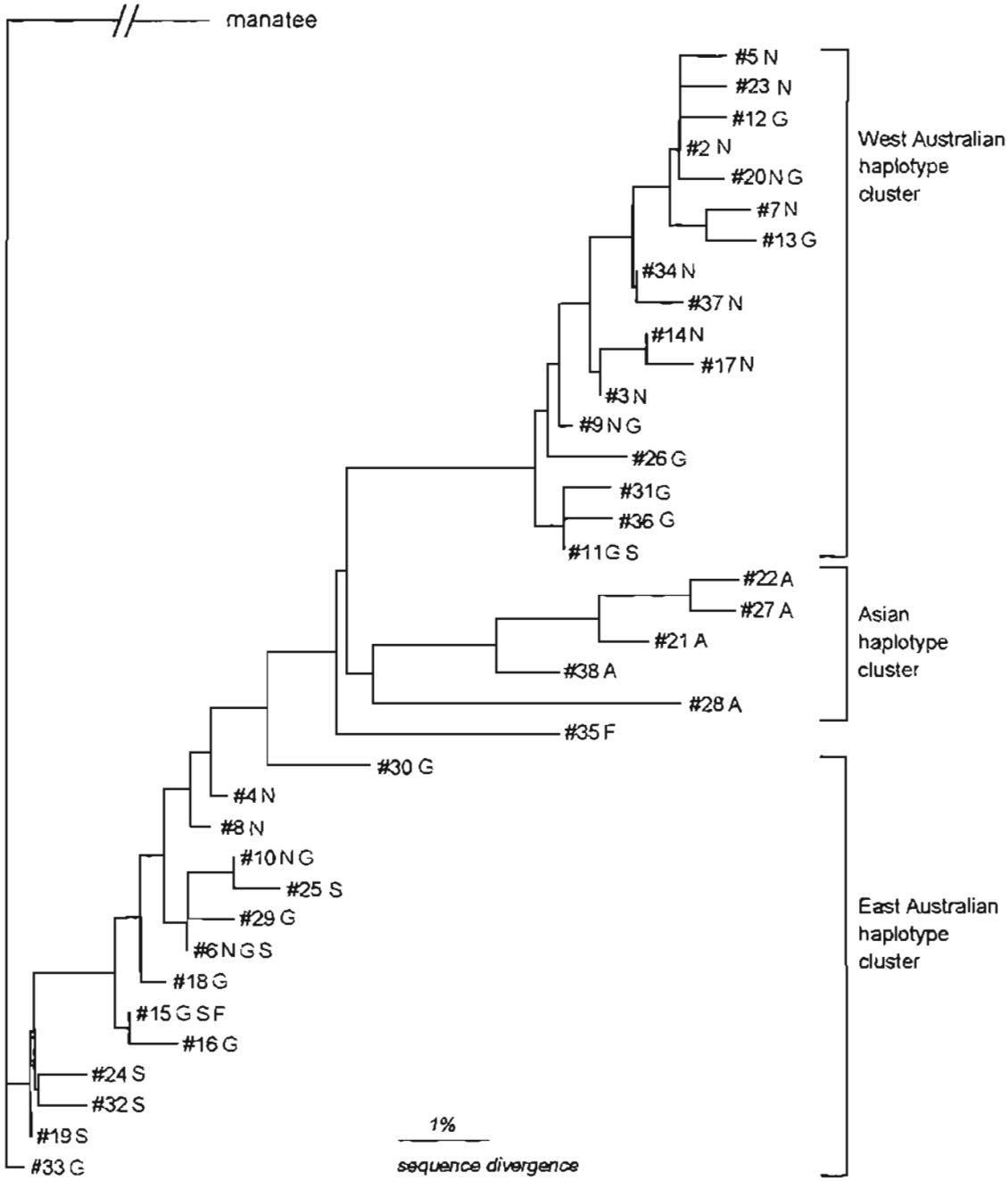

Figure 7.6: PHYLIP Neighbour Joining tree. Three main haplotype clusters are evident: 1) West Australian, 2) East Australian, and 3) Asian. Numbers at the terminals (preceded by #) refer to haplotypes as listed table 7.1. Next to haplotypes are the nominated geographic units abbreviated as follows: N, North & West Australia; G, Great Barrier Reef; S, South East Queensland; F, West Indian Ocean; and A, Asian.

Three main clusters of haplotypes are prominent: **West Australian**, **East Australian**, and **Asian** (figure 7.3). The West Australian haplotype cluster is more deeply diverged relative to the East Australian and Asian cluster. Bootstrap values are shown at the nodes of the consensus tree (figure 7.3) showing the most consistently defined haplotype clusters. After the removal of the outgroup, a tree was constructed by PAUP to illustrate the three clusters to greater effect (figure 7.4). A bootstrap analysis of this data set was constructed by PAUP, and the bootstrap values at the nodes of the main branches of figure 7.4 indicated the most consistently defined haplotype clusters. The distribution of the haplotype clusters is shown in figure 7.5.

7.2.2 PHYLIP

The PHYLIP Neighbour Joining tree (figure 7.6) has a similar topology to the PAUP gene tree (figure 7.3), in that they both have the same three main clusters of haplotypes (West Australian, East Australian and Asian), with slight rearrangements of the terminal branches (haplotypes) within each cluster.

7.2.3 AMOVA

Dugong populations were grouped into a variety of different geographic units on the basis of the haplotypes within those populations and geography (table 7.2 and figure 7.5). These groups were tested for their significance via a series of AMOVA tests (table 7.3). The population and haplotype numbers in table 7.2 are used consistently throughout the text. The absence of genetic subdivision is the null hypothesis for all of these tests. The term 'panmixis' will be used for convenience throughout this chapter to imply lack of genetic structure. The logic of the progression of the AMOVA tests (refer to figure 7.1 for definition of the various ϕ-statistics) is explained below:

Tests 1 and *2* investigated the probability of significant levels of genetic subdivision globally and among Australian populations respectively. There were no ϕ-statistic values for 'among groups' (ϕ_{CT}), or 'among populations within groups' (ϕ_{SC}) as there was only one group. The 'within populations' (ϕ_{ST}) indicated that the null hypothesis of panmixis was rejected both at the global scale (*Test 1*) and within Australian waters (*Test 2*).

Table 7.3: AMOVA (Version 1.55) analysis. Observed among groups, AG (ϕ_{CT}), among populations within group, AP/WG (ϕ_{SC}) and within populations, WP (ϕ_{ST}) values. Significance values were based on 1000 permutations. Refer to table 7.2 and figure 3.2 for details on the location of the listed population numbers. Region sample sizes are indicated in italics within brackets.

AMOVA tests indicating the relevant group & population files	Variance Component	% Total Variance	ϕ-Statistics	p values ($\alpha \leq 0.05$)
Test 1) 1 group, **24** (all) populations. Test for universal panmixis (*103* individuals). 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24	AP	37.68	$\phi_{ST} = 0.377^{*}$	<0.0010
	WP	62.32		
Test 2) 1 group, **18** (Australian) populations. Test for panmixis among Australian dugongs (*92* ind.). 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24	AP	27.80	$\phi_{ST} = 0.278^{*}$	<0.0010
	WP	72.20		
Test 3) 3 groups, **4** (Australian > 15 ind.) populations. 11 (Torres Strait, *21* ind.) 17 (Shoalwater Bay, *15* ind.) 19 20 (Hervey & Moreton Bay, *19* ind.)	AG	29.49	$\phi_{CT} = 0.295^{*}$	<0.0010
	AP/WG	-4.59	$\phi_{SC} = -0.065^{NS}$	0.3167
	WP	75.10	$\phi_{ST} = 0.249^{*}$	<0.0010
Test 4) 3 groups, **18** (Australian) populations. 7 8 9 10 11 (North & West Australian, *29* ind.) 12 13 14 15 16 17 18 (GBR, *37* ind.) 19 20 21 22 23 24 (SE Qld, *26* ind.)	AG	26.64	$\phi_{CT} = 0.266^{*}$	0.0030
	AP/WG	5.99	$\phi_{SC} = -0.082^{NS}$	0.0939
	WP	67.37	$\phi_{ST} = 0.326^{*}$	<0.0010
Test 5) 5 groups, **24** (all) populations. 1 2 3 4 (Asian, 7 ind.) 5 6 (W.Ind-Ocean, *4* ind.) 7 8 9 10 11 (North & West Australian, *29* ind.) 12 13 14 15 16 17 18 (GBR, *37* ind.) 19 20 21 22 23 24 (SE Qld, *26* ind.)	AG	35.42	$\phi_{CT} = 0.354^{*}$	<0.0010
	AP/WG	6.67	$\phi_{SC} = 0.103^{*}$	0.0380
	WP	57.91	$\phi_{ST} = 0.421^{*}$	<0.0010
Test 6) 4 groups, **20** (all except Asia) populations. 5 6 (W.Ind-Ocean, *4* ind.) 7 8 9 10 11 (North & West Australian, *29* ind.) 12 13 14 15 16 17 18 (GBR, *37* ind.) 19 20 21 22 23 24 (SE Qld, *26* ind.)	AG	28.47	$\phi_{CT} = 0.285^{*}$	<0.0010
	AP/WG	4.95	$\phi_{SC} = 0.069^{NS}$	0.1179
	WP	66.58	$\phi_{ST} = 0.334^{*}$	<0.0010
Test 7) 4 groups, **22** (all except W.Ind-Ocean) populations. 1 2 3 4 (Asian, 7 ind.) 7 8 9 10 11 (North & West Australian, *29* ind.) 12 13 14 15 16 17 18 (GBR, *37* ind.) 19 20 21 22 23 24 (SE Qld, *26* ind.)	AG	34.57	$\phi_{CT} = 0.346^{*}$	<0.0010
	AP/WG	7.50	$\phi_{SC} = 0.115^{*}$	0.0280
	WP	57.93	$\phi_{ST} = 0.421^{*}$	<0.0010
Test 8) 4 groups, 4 pop. (West Aust. haplotypes only) 11 (Torres Strait, *12* ind.) 15 (Townsville, *6* ind.) 17 (Shoalwater Bay, *5* ind.) 19 (Hervey Bay, *7* ind.)	AG	37.18	$\phi_{CT} = 0.372^{*}$	<0.0010
	AP/WG	0.00	$\phi_{SC} = 0.000^{NS}$	<0.0010
	WP	62.82	$\phi_{ST} = 0.372^{*}$	<0.0010
Test 9) 5 groups, 5 pop. (East Aust. haplotypes only) 11 (Torres Strait, *9* ind.) 15 (Townsville, *3* ind.) 17 (Shoalwater Bay, *10* ind.) 19 (Hervey Bay, *6* ind.) 20 (Moreton Bay, *12* ind.)	AG	24.14	$\phi_{CT} = 0.241^{*}$	<0.0010
	AP/WG	0.00	$\phi_{SC} = 0.000^{NS}$	<0.0010
	WP	75.86	$\phi_{ST} = 0.241^{*}$	<0.0010

^{NS} failure to reject the null hypothesis of 'no restriction to gene flow'.
* reject the null hypothesis of 'no restriction to gene flow'.

Test 3 included those Australian populations with substantial sample sizes (15 - 21 individuals), of which there were three groups represented by four populations: Torres Strait (21 individuals), Shoalwater Bay (15 individuals), Hervey and Moreton Bays (19 individuals combined). This test indicated no restriction to gene flow 'among populations within groups', but did indicate restriction to gene flow among the nominated groups.

Test 4. The other (minor) Australian populations were pooled with the (major) Australian populations into the three groups on the basis of geography (see table 7.4 for names of these nominated geographic units). There was an absence of 'within group' substructure (ϕ_{SC} see table 7.3). However, as for *Test 3*, there was restriction to gene flow 'among groups'. Therefore, these nominated groups appear to be representative of the actual genetic substructure along the Australian coast.

The Asian and West Indian Ocean groups were then included with the Australian groupings (of *Test 4*) to form a total of five groups (*Test 5*). The borderline rejection of the null hypothesis of gene flow 'among populations within groups' (ϕ_{SC}), suggests substructure within-groups (as did the rejection of the 'among populations' and 'among groups' ϕ-statistics). For this reason, *Tests 6 & 7* were carried out to investigate whether the Asian or West Indian Ocean groups introduced the within-group substructure.

The null hypothesis of gene flow 'among populations within groups' (ϕ_{SC}) was accepted in *Test 6*, which included the groupings of *Test 5* minus Asia. That is, there was no within-group substructure for the West Indian Ocean group. This conclusion must be interpreted with caution given the small sample size of the West Indian Ocean.

Test 7 corresponded to *Test 6*, except that the West Indian Ocean group was excluded instead of the Asian group. The null hypothesis for 'among populations within groups' (ϕ_{SC}) was rejected suggesting that it was the Asian group that provided the substructure, as *Tests 3 & 4* indicated substructure did not exist within the nominated Australian groups.

West and East Australian haplotype clusters are obviously divergent (figures 7.3 & 7.4) suggesting historical separation with recent geographic overlap. *Tests 8* and *9* were designed to examine genetic subdivision within each haplotype cluster separately. Each group was represented by

one population from the east coast of Queensland. The nominated groups/populations included: Torres Strait, Townsville, Shoalwater Bay, Hervey and Moreton Bay. Moreton Bay was not included in *Test 8* as haplotypes of the West Australian cluster were not observed in this population. Populations with small sample sizes (less than 7) were also excluded. *Test 9* included the East Australian haplotype cluster only, whereas *Test 8* included the West Australian haplotype cluster only (see figure 7.4). Both *Tests 8* and *9* indicate a restriction to gene flow 'among groups' (ϕ_{CT}), that is, genetic subdivision exists within an Australian haplotype cluster.

Table 7.4: Haplotype clusters and geographic units. Haplotype clusters are evident in figure 7.4. The two Australian haplotype clusters overlap geographically in the region of the Great Barrier Reef. Support for the populations grouped in the geographic units is summarised in table 7.3.

Region	*Haplotype cluster*	*Geographic Units*
Australia	West Australian East Australian	North & West Australian Great Barrier Reef South East Queensland
Asian	Asian	Asian
West Indian Ocean	No haplotype cluster was evident (a result which may be confounded by the small sample size for this region).	West Indian Ocean

As I performed nine AMOVA tests on the same set of data (or subsets thereof), there is the potential for increasing the probability of making a type I error. To accommodate this potential, a conservative approach is employed in which one lowers the type I error of the statistic of significance for each comparison, so that the probability of making any type I error at all in the entire series of tests doesn't exceed α. This probability is called the 'experimentwise error rate' (Sokal & Rohlf, 1995). The adjusted level of significance for comparison with each critical value was obtained from the following equation (Sokal & Rohlf, 1995):

$$\alpha' = 1 - (1 - \alpha)^{1/k}$$

Where α is the significance level, and k is the number of statistical tests. That is, for nine AMOVA tests at a level of $\alpha \leq 0.05$:

$$\alpha' = 1 - (1 - 0.05)^{1/9}$$
$$= 0.006$$

The critical value from each AMOVA test listed in table 7.3 was then reconsidered in comparison to the adjusted α. All of the conclusions remained the same with the exception of tests 5 and 7, in which there was failure to reject the null hypothesis of gene flow 'among populations within groups' (ϕ_{SC}), suggesting a lack of substructure within-groups at the $\alpha = 0.006$ level. This correction does not alter the conclusions greatly, except that it provides additional support for the suggested five groupings.

7.2.4 MacClade

The minimum number of migration steps allowed by the phylogenetic tree seen in figure 7.7, was nine (ten when the outgroup was included). If the five groups were part of a single panmictic population, the observed number of inter-group migration events should fall within the probability distribution constructed from 1000 random joining/splitting trees (Slatkin & Maddison, 1989; Maddison & Slatkin, 1991). However, the observed number of steps was not contained within this distribution (figure 7.8). That is, only nine interlocality migrations on the reconstructed tree would be highly unlikely under panmixis, suggesting restriction to gene flow among the five nominated groups.

When the Asian and West Indian Ocean samples were removed from the data set and the phylogenetic tree and distribution regenerated, the minimum number of migration steps allowed by the tree was seven (data not shown). This value fell outside of the probability distribution constructed from 1000 random joining/splitting trees (data not shown). That is, only seven interlocality migrations on the reconstructed tree would be highly unlikely under panmixis, suggesting restriction to gene flow among the three Australian nominated groups.

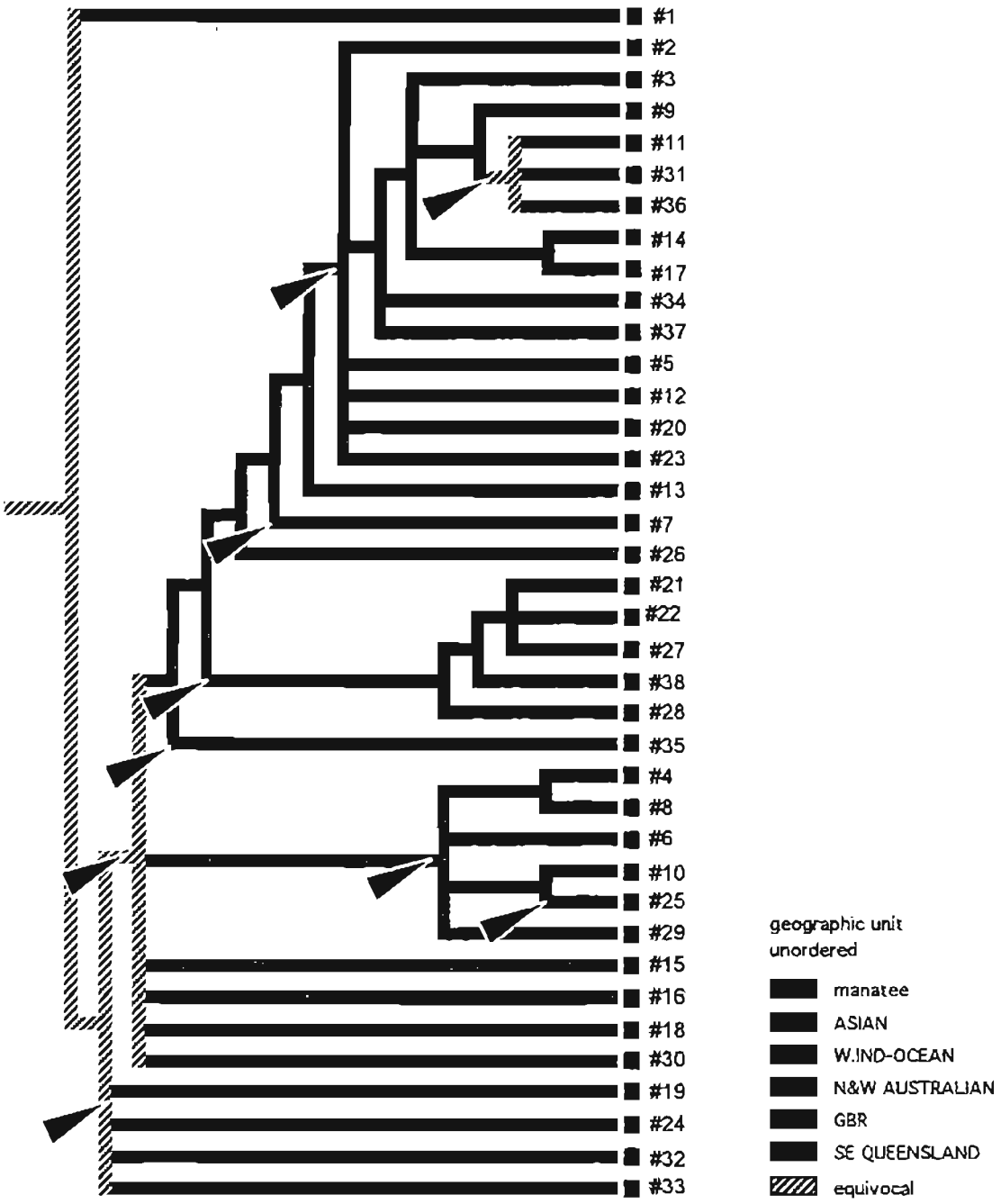

Figure 7.7: A MacClade group traced tree. The nine inter-group migration events (ten if including the outgroup) required to explain the geographic distribution of haplotypes are highlighted by the arrows. The numbers on the branch terminals refer to haplotypes as listed in table 7.1. Equivocal patterned branches indicate where more than one state (type of nucleotide change) can be constructed with equal parsimony at a branch.

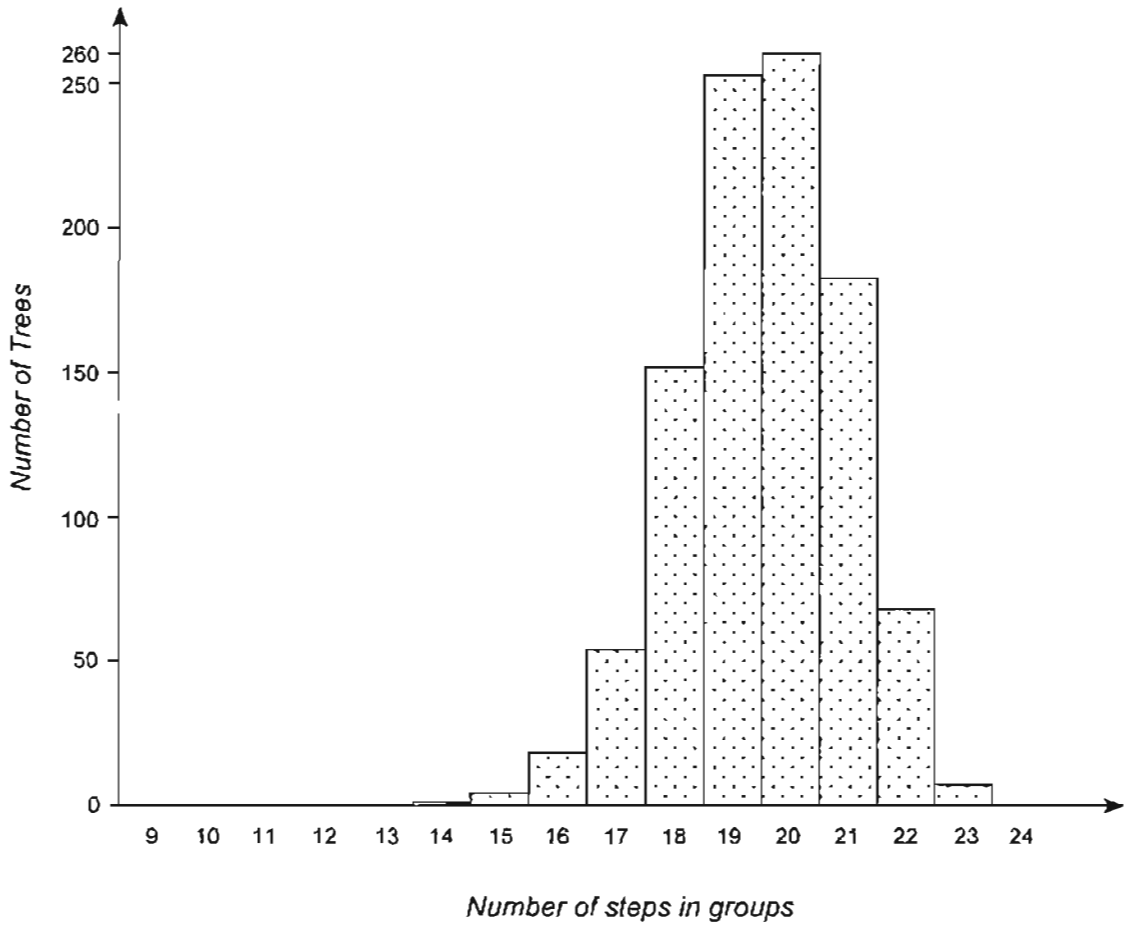

Figure 7.8: Probability distribution. The probability distribution was constructed from 1000 random joining/splitting trees created by MacClade. The number of intergroup migration steps allowed by a group-traced tree (figure 7.7) was nine (omitting the outgroup), a number of steps which falls outside of the probability distribution, indicating that there is restriction to gene flow among groups.

7.2.5 REAP

As MONTE (REAP, McElroy *et al.*, 1992) is used to investigate haplotype frequency rather than haplotype similarity, it was not appropriate for the large number of rare haplotypes in this data set. However, even though no information regarding genetic structure was obtained from the MONTE program, the MONTE input was required as part of the input file to DA analysis.

Table 7.5: Genetic diversity within the geographic units. The within-geographic unit haplotype and nucleotide diversity was calculated using the program DA. The numbers shown with each geographic unit correspond to the populations as indicated in table 7.2 and figure 3.2. Standard errors for haplotype diversity are indicated within brackets. No standard errors exist for nucleotide diversity within-geographic unit measures, as these are absolute values.

Geographic unit	*Sample size*	*Haplotype diversity*	*Nucleotide diversity*
Asian 1 2 3 4	7	0.9048 (+/- 0.10330)	0.037819
West Indian Ocean 5 6	4	0.5000 (+/- 0.26517)	0.025450
N&W Australian 7 8 9 10 11	29	0.9113 (+/- 0.03142)	0.044123
GBR 12 13 14 15 16 17 18	37	0.8964 (+/- 0.03008)	0.047661
SE Qld 19 20 21 22 23 24	26	0.6154 (+/- 0.10454)	0.016175
	Average	0.7656 (+/- 0.00754)	0.034246 (+/- 0.0000347)

Nucleotide divergence and nucleotide diversity within the five geographic units (as in AMOVA *Test 5*, see table 7.3) were calculated using the program DA (REAP, McElroy *et al.*, 1992), from which N_{ST} values were calculated and presumed migration rates (Nm) estimated (Chapter 8). Table 7.5 indicates low haplotypic diversity in the West Indian Ocean, and a relatively low haplotypic diversity in the South East Queensland (SE Qld) geographic unit compared to the other Australian geographic units (table 7.5); a relatively high diversity was observed for Asian dugongs. Some of the estimates of haplotype and nucleotide diversity may be confounded by small sample sizes, and they should be interpreted with caution. In addition, high genetic diversity within the Asian geographic unit may be evidence of undetected

genetic subdivision. The relevance of the genetic diversities within each geographic unit is discussed in Chapters 11 and 12.

7.2.6 NDA

The Nucleotide Diversity Analysis (Holsinger & Mason-Gamer, 1996) was designed to partition diversity into hierarchical components while allowing any structure present in the data to 'emerge naturally'. With the exception of the dugong populations of the West Indian Ocean, no such correlation between haplotype clustering and geographic regions is suggested by figure 7.9.

7.3 DISCUSSION

7.3.1 Sequence characteristics

The observed transition to transversion ratio and the directional pressure against the base guanine, between the dugong and manatee HVR-1 DNA sequences, are consistent with substitution biases reported from mtDNA of other mammals in both the coding and non coding region of mtDNA (Brown *et al.*, 1982; Greenberg *et al.*, 1983; Perna & Kocher, 1995b). This indicates that changes among all bases are not equally likely (unordered), which was the chosen option/assumption in the PAUP analyses (section 7.1.1). The alternative option in PAUP, would have been to specify characters as ordered. That is, to assume that a particular base was ancestral at each site. Characters are more unordered than ordered because it is assumed that mutations occur by chance, which is why that option was chosen. Transversions can be weighted in PAUP analyses, but as only one transversion was observed among the 37 haplotypes sequenced, it was treated with the same weighting as the transitions.

The high transition to transversion ratio observed among the dugong haplotypes, and a suggested rate variation (i.e., presence of substitution hot spots) within HVR-1, indicated a requirement for correction to accommodate this pattern of substitution. An appropriate DNA evolution model was investigated and is reported in Chapter 8. As a consequence, whenever a distance matrix was required for analysis, a Tamura-Nei (Tamura & Nei, 1993) corrected distance matrix was employed (see Chapter 8).

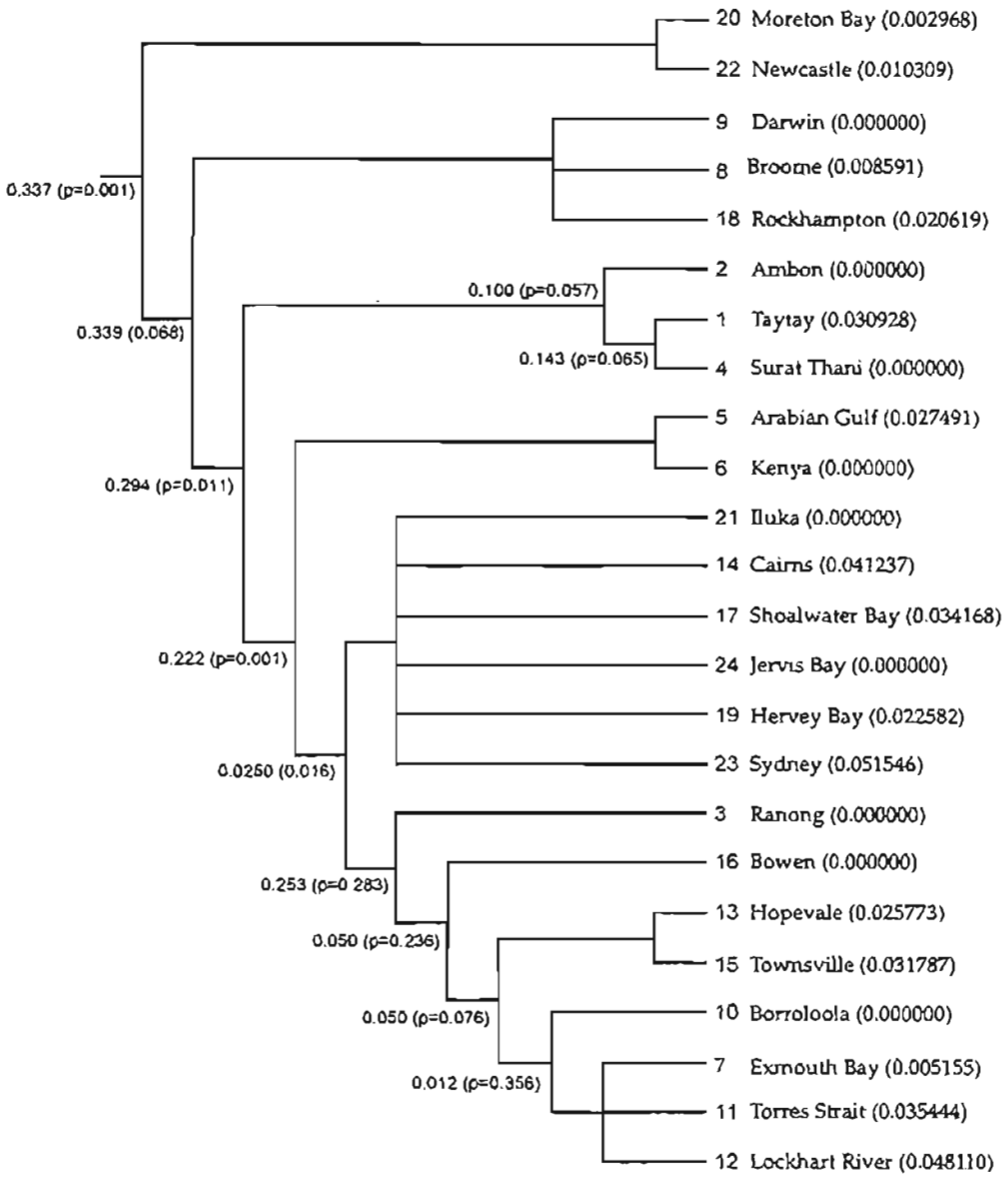

Figure 7.9: Hierarchical analysis of haplotype diversity for the dugong. Populations and their numbers correspond to those in table 7.2, with nucleotide sequence diversity of each population contained in parentheses, estimated following Nei & Tajima (1981) via NDA. The number at each node is the distance between its two daughter nodes, and the *p*-value reported is the probability of obtaining a distance that is as great or greater under the null hypothesis of no differentiation between the daughter nodes (based on 10,000 resamplings).

7.3.2 Intraspecific genetic subdivision

7.3.2.1 *Haplotype clusters*

With the exception of the West Indian Ocean dugong populations, no correlation between haplotype clustering and geographic regions was recognised from the Nucleotide Diversity Analysis (NDA, section 7.2.6, figure 7.9). The failure of NDA to infer phylogeographic units may be attributed to the large number of rare haplotypes, the extensive overlap in the geographic range of many haplotypes, and the small sample size of some populations. Small sample sizes hinder the effectiveness of an algorithm heavily dependent on haplotype frequencies. A successful example of the NDA to illustrate genetic subdivision correlated with geographic distribution, is presented by Merila *et al.* (1997). Using mtDNA control region haplotypes as a genetic marker, Merila *et al.* (1997) sampled 10 to 25 individuals from 10 Greenfinch (*Carduelis chloris*) populations, which were characterised by low within-population genetic diversity. NDA detected two major genetic units among the Greenfinch populations, explained by explosive post-Pleistocene recolonisation of northern Europe from the south (Merila *et al.*, 1997).

Correlated clustering of haplotypes with geographic regions was better revealed by the construction of the PAUP and PHYLIP gene trees, which consider similarity of the nucleotide sequences only. According to the strict definition of an Evolutionarily Significant Unit (ESU) of Moritz (1994a; 1994b) and Moritz *et al.* (1995), there is one strongly supported monophyletic cluster on the mtDNA haplotype phylogram (figures 7.3, 7.4 & 7.6). Therefore, there is only one ESU among all of the dugong samples. Within the single ESU, three major haplotype clusters are obvious and consistent: West Australian, East Australian, and Asian. Another feature consistent and apparent among the gene trees is a deep split separating the West Australian haplotype cluster from the East Australian and Asian haplotype clusters. This indicates a closer relationship between the Asian and East Australian dugongs, relative to the West Australian dugongs.

The geographical distribution of the two Australian haplotype clusters (figure 7.5), suggests the Torres Strait may have acted as a barrier to dugong movement during phases of low sea level in the Pleistocene. These haplotype clusters have overlapped along the Great Barrier Reef perhaps only during the Holocene when the Torres Strait last became a seaway about

6000 years ago (see Chapter 11 for further discussion on this). Whether representatives of the two clusters interbreed can only be clarified with the application of a nuclear genetic marker. Estimation of possible divergence times for the haplotype clusters is introduced in Chapter 8, and a model of the historical phylogeography and possible barriers to gene flow, is discussed in Chapter 11.

7.3.2.2 *Dugong samples of the New South Wales coast*

Hervey Bay is assumed to be the originating population for dugongs necropsied in New South Wales (NSW) because of their genetic similarity and the time of sample collection. With the exception of sample ORC139, all of the samples collected beyond the dugong's recognised range on the south east coast of Australia (i.e., New South Wales), were typical of East Australian haplotypes. The NSW dugongs were necropsied between September 1992 and January 1993 (Appendix C), coinciding with population reduction in Hervey Bay (Preen & Marsh, 1995). The Hervey Bay population was severely reduced from an estimated 1753 (s.e.±388) in 1988, to 71 (s.e.±40) dugongs in 1992. Large scale flooding of the river feeding the bay in early 1992 caused the death of the sea grass beds by sedimentation, depriving dugongs of their food source (Preen & Marsh, 1995). The reduction in the size of the Hervey Bay dugong population is believed to have resulted from dugongs dying of starvation or leaving the area.

Sample ORC139 was necropsied in 1994 and is assumed to be from Hervey Bay because it has a West Australian haplotype, and also had the same haplotype (H#11, table 7.2) as another dugong sampled from Hervey Bay (sample HB5, table 7.2). H#11 was the only West Australian haplotype that penetrated as far southeast as Hervey Bay.

7.3.2.3 *Geographic and genetic units*

From the HVR-1 sequenced in 103 dugongs, AMOVA and MacClade analyses both support the following five geographic units for the dugong (table 7.3 & figure 7.10): 1) **Asian** (Ranong, Surat Thani, Taytay, Ambon), 2) **West Indian Ocean** (Arabian Gulf, Kenya), 3) **N&W Australian** (Exmouth, Broome, Borroloola, Darwin, Torres Strait), 4) **GBR** (Lockhart River, Starcke, Cairns, Townsville, Bowen, Shoalwater Bay & Rockhampton) and 5) **SE Qld** (Moreton & Hervey Bays and samples collected beyond the dugong's range on the coast of NSW).

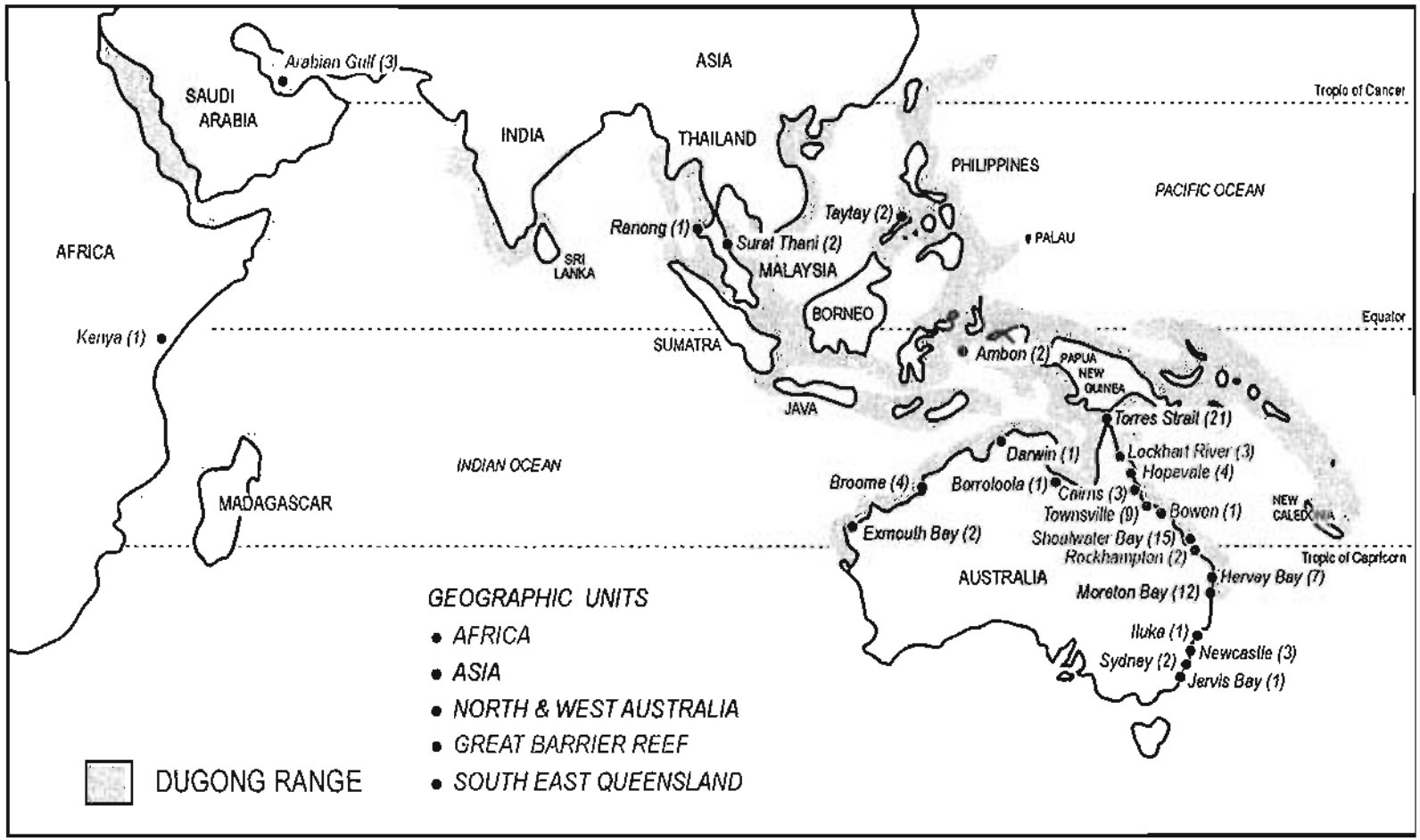

Figure 7.10: Dugong range and nominated geographic units. Sample size of each population is indicated within brackets.

Only the Asian samples constitute a 'phylogeographic unit' in that they are genetically as well as geographically distinct. Geography was the primary basis for determining the West Indian Ocean populations as a single geographic unit, but they are genetically similar to the East Australian haplotype cluster, and actually share one haplotype with Australian populations (haplotype #15, which is the most common haplotype - see figure 7.2) (see Chapter 11 for discussion on this). Within Australia, there is broad overlap of haplotypes along the east Australian coast. Genetic substructure exists within each haplotype cluster in a manner that is suggestive of an isolation-by-distance distribution.

To circumvent the problem of small sample size for some populations, I pooled these populations with their neighbours. Examining geographic heterogeneity in population frequency distributions was initially attempted through a Monte Carlo simulation (McElroy *et al.*, 1992; Roff & Bentzen, 1989) using the program MONTE (REAP, McElroy *et al.*, 1992). This program was expected to cope with the low sample sizes from many of the populations in the data set. However, because there were so many rare haplotypes, the attempts to use MONTE analyses failed. A haplotype is considered 'rare' when it has a frequency of one per population (e.g., refer to figure 7.2 and table 7.2). The occurrence of rare haplotypes was overestimated because of the small number of samples taken from some populations. The high occurrence of rare haplotypes suggested that: 1) the populations were not sampled adequately and/or 2) a healthy amount of genetic variation exists within the dugong populations and geographic units. An increase in sample size to at least 15, and preferably 20, samples per population, should detect finer scale genetic substructure in the dugong.

7.3.2.4 Genetic support for morphological differences among Australian dugong populations

Although the sample size representing the Gulf of Carpentaria is minimal (i.e., one representative from Borroloola) the proposed genetic partitioning supports the findings of Spain & Marsh (1981) who found geographic variation in skull morphometrics of dugongs from Townsville (19°15'S, 146°45'E) and Wellesley Island (16°41'S, 139°8'E).

7.3.3 Nuclear copies of mitochondrial genes

Some of the features in the topology of the mtDNA haplotype gene trees may be considered as unusual. These are 1) the high similarity of West Indian Ocean dugongs to those of the East Australian haplotype cluster, and 2) the distinction of the West Australian haplotype cluster. For this reason, I considered the possibility that nuclear copies of mtDNA may exist. The occurrence of nuclear copies of mtDNA was documented more than ten years ago (e.g., Fukuda *et al.*, 1985). Nuclear copies evolve more slowly, and their mode of inheritance is more variable than their mitochondrial counterparts (Zhang & Hewitt, 1996; Perna & Kocher, 1996). For these reasons, nuclear copies will confound population genetic analyses when included in the mitochondrial data set, and phylogenetic analyses (Zhang & Hewitt, 1996), as conclusions based on coalescent analysis would certainly be misleading. Recently, the confounding effects of nuclear copies on population studies using mtDNA have been readdressed and emphasised in reviews by Zhang & Hewitt (1996) and Perna & Kocher (1996). However, if there are nuclear copies recognised in a mitochondrial data set, their presence would not be entirely negative. As nuclear copies evolve more slowly than their corresponding authentic mitochondrial sequences ('molecular fossils'), they can be effective as outgroups to mtDNA in constructing gene trees, particularly when no other suitable outgroup can be found (e.g., Zischler *et al.*, 1995).

Listed in table 7.6 are five criteria summarised by Zhang & Hewitt (1996) that can alert the researcher to the possible existence of nuclear copies. Little support for the presence of nuclear copies is provided by the data in this study. The suspected presence of nuclear copies may also be counter-argued by the haplotype clusters being well associated with a historical phylogeography model presented in Chapter 11.

Amplification of nuclear copies can be avoided by maximising selective amplification of mtDNA by using 1) enriched or purified mtDNA, 2) a combination of several primers, and 3) target-specific primers (which requires full characterisation of both the mtDNA and nuclear DNA counterparts). Data produced from these methods are 'fairly free' from errors resulting from nuclear pseudogenes (Zhang & Hewitt, 1996). The level of stringency of PCR conditions and the hierarchal approach to obtaining product for sequencing for the broad survey are detailed in section 4.3.3. The use of enriched or purified mtDNA was not a method employed

in this study. However, a combination of primers was used to amplify and sequence the control region (figure 6.1), and species-specific primers were used to amplify HVR-1, which was the region of DNA routinely sequenced for the broad scale survey (see section 6.3.1).

Table 7.6: Situations when the existence of nuclear copies should be considered, and the respective observations obtained from this study. The five situations when a researcher should seriously consider the possibility of nuclear copies of mitochondrial sequences are listed by Zhang & Hewitt (1996). Only in one situation, does the data obtained from this study suggest the possible existence of nuclear copies of mitochondrial DNA.

Reason to suspect nuclear copies	Observed?
1) PCR amplification constantly produces more than one band or different bands.	No
2) Sequence ambiguities or background bands persist.	No
3) Unexpected deletions/insertions, frameshifts or stop codons occur.	No
4) Nucleotide sequences obtained are radically different from those expected.	No
5) Phylogenetic analysis yields an unusual or contradictory tree topology.	The phylogenetic analysis revealed an unexpected tree topology.

7.3.4 Comparison between dugong and West Indian manatee genetic structure

Garcia-Rodriguez *et al.* (in press) recently completed a study examining the genetic structure of the West Indian manatee (*Trichechus manatus*). The HVR-1 segment of the control region was sequenced from 86 individuals of the West Indian manatee and 16 individuals of the Amazonian manatee (*T. inunguis*). A total of 15 mtDNA haplotypes were found for the West Indian manatee, and an additional eight for the Amazonian manatee, with a total of 51 polymorphic sites. Garcia-Rodriguez *et al.* (in press) found complete sequence homogeneity for the West Indian manatee around the Florida peninsula. The findings of homogeneity among manatees of

Florida by Garcia-Rodriguez *et al.* (in press) is supported by the low sequence diversity in 225 bp of the cytochrome *b* gene among four West Indian manatees (Bradley *et al.*, 1993; Ozawa *et al.*, 1997; figure 5.4). However, despite the homogeneity across geographic regions of Florida, an allozyme study of 59 Florida manatees indicated a polymorphism of 0.300 and heterozygosity of 0.050, which is comparable to most land mammals (McClenaghan & O'Shea, 1988; table 2.2).

The overall haplotype and nucleotide diversity within the West Indian manatee was relatively high (0.835 and 0.0414 respectively) (Garcia-Rodriguez *et al.*, in press). This compares to the haplotypic (0.7656) and nucleotide diversity (0.0343) of the dugong (table 7.5). In conclusion, sirenians appear to have relatively high intraspecific diversity.

Both the West Indian manatee and the dugong have a coastal linear distribution, although the populations of the West Indian manatee are probably less continuous than the dugong's. Unsuitable habitat (e.g., along the coast of Venezuela) including lack of fresh water, limit manatee distribution. The distribution of gene types of the dugong is suggestive of an isolation-by-distance model, whereas I predict the West Indian manatee may be more reflective of a stepping stone model.

7.4 CONCLUSIONS

There is significant restriction to gene flow both globally and among Australian dugong populations. The haplotypes around the Australian coast are distributed in a fashion suggestive of an isolation-by-distance model. From the HVR-1 of mtDNA, three haplotype clusters were recognised: 1) **West Australian**, 2) **East Australian**, and 3) **Asian**. The two Australian mtDNA haplotype clusters overlap in the geographic region of the Great Barrier Reef. Haplotypes of West Indian Ocean dugongs are very similar to East Australian haplotypes. The geographical range of Asian mtDNA haplotypes does not overlap with Australian or West Indian Ocean haplotypes. Within each haplotype cluster along the east Australian coast, there is genetic substructure suggestive of an isolation-by-distance distribution of haplotypes.

CHAPTER EIGHT

MUTATION RATES, DIVERGENCE TIMES, FEMALE MIGRATION RATES & EFFECTIVE FEMALE POPULATION SIZE

The objective of this chapter is to assess the nature of base substitutions in the hypervariable region 1 (HVR-1) of mtDNA. The Tamura-Nei algorithm (Tamura & Nei, 1993) was deemed the most suitable to accommodate the pattern of substitution. Determination of a substitution rate enabled divergence times among haplotype clusters and geographic units to be estimated (defined in Chapter 7). Divergence times were then used in the development of a phylogeographic model for the dugong (presented in Chapter 11). Additional calculations included estimates of the effective female population size of each geographic unit, and calculations of gene flow (permanent female exchange). The estimates are tentative due to the small sample sizes from some populations.

8.1 PATTERN and RATES OF SUBSTITUTION

Substitution rates can be estimated from gene trees dated in absolute terms on the basis of extrinsic information. Usually palaeontologically determined dates for one or more of the branchpoints of the tree are employed. All of the other dates are then extrapolated in proportion to the lengths of the various branches. Using the calculated substitution rate, divergence times among genetically distinct groups, subspecies and species can often be mapped to historical geological events (Chapter 11). The reliability of the calibration date is crucial, as is the assumption of a steady rate of evolution, or 'molecular clock' (Avise, 1994).

If the assumption of a constant rate of change is not valid, the resultant error could result in divergence times being wrongly estimated by many millions of years. I consider that Rainey *et al.* (1984) are likely to have made such an error in estimating divergence times among the sirenians in their immunological comparisons using antisera against: 1) bone extracts of

Steller's sea cow and all four extant sirenian species, and 2) serum albumins of the dugong, the Florida manatee, and the Indian and the African elephants. Using a palaeontological reference for the divergence time between elephants and sirenians of 50 to 60 Myr ago (Palaeocene), Rainey *et al.* (1984) calibrated divergence times between the sirenians on the basis of their immunological differences. Rainey *et al.*'s (1984) assumption of constant mutation rate is probably invalid, as the elephant has recently been recognised as having a faster mutation rate than sirenians and other mammals, at least in the cytochrome *b* (Irwin & Arnason, 1994; Ozawa *et al.*, 1997) and 12s mtDNA (Lavergne *et al.*, 1996) genes. Under these circumstances, the assumption of a constant rate of mutation between elephants and sirenians would have the effect of underestimating the divergence times between the sirenians. Rainey *et al.* (1984) dated the divergence between dugongs and manatees at between 17 and 20 Myr ago (lower Miocene). Fossil data provides a divergence time of 30 to 40 Myr (upper Eocene to lower Oligocene) (Domning, 1994). Domning (1994) showed that according to fossil evidence, the divergence between dugongs and manatees could be no later than the upper Oligocene (>25 Myr ago).

It is possible that evolutionary rates also differ among sirenian species (Domning, 1982). The Amazonian manatee has a greater number of derived morphological characters, compared with other manatees (Domning, 1982). Whether the greater number of derived morphological characteristics of the Amazonian manatee is reflected in its mutation rate remains to be demonstrated. However, the implications of varying mutation rates among sirenians on estimated divergence times should be considered.

8.2 CHOICE OF SUBSTITUTION MODEL

As well as varying rates of mutation among species, within-genome variation in substitution patterns further complicates an accurate measurement of substitution rate. Substitution rates are estimated by observing the number of substitutions among compared sequences. However, simple pairwise comparisons may not elucidate the actual substitution processes leading to the observed divergences. Several models are available to accommodate various patterns of DNA sequence evolution. The simplest of these was proposed by Jukes & Cantor (1969), and assumes

that all nucleotide sites have an equal probability of substitution. However, HVR-1 of mtDNA has a complicated pattern of nucleotide substitution, including a high ratio of transitions to transversions (Brown *et al.*, 1982; Aquadro & Greenberg, 1983) and unequal rates of substitution (Kocher & Wilson, 1991). In addition, transitional changes between purines and between pyrimidines in the HVR–1, occur with different frequencies (Tamura & Nei, 1993). Kimura's two-parameter model (Kimura, 1980) and the model of Tamura & Nei (1993), both accommodate differing rates of transitions and transversions. The latter approach also accommodates variable substitution rates among nucleotide sites and biases in substitution type (Tamura & Nei, 1993). To decide which model was the most appropriate to estimate mutation rates, I investigated whether substitution bias and among-site substitution rate differences existed in the HVR–1 of mtDNA as outlined below.

8.2.1 Transition:Transversion ratio

To determine if there are differing rates in the occurrence of transitions (ts) and transversions (tv), ts:tv ratios were calculated from the pairwise comparisons of haplotypes using MEGA (Kumar *et al.*, 1993). The number of transitions are expected to be higher than the number of transversions among the most closely related haplotypes (Aquadro & Greenberg, 1983; Brown, 1985). Comparison of the polymorphic sites in dugong HVR-1 sequences with the Florida manatee, showed a ts:tv ratio of one (table 8.1). Within-species (i.e., dugong) comparison of the HVR-1 haplotypes polymorphic sites, indicated the observed frequency of transversions was very low at 0.5% (table 8.1). The observed ts:tv ratios are presumed to underestimate the actual number of transitions, as backmutations (which are not observed) are likely to have occurred.

An estimate of the ts:tv ratio of 66:1 was obtained from one of the 41 equally parsimonious trees (excluding the outgroup) calculated by PAUP (Swofford, 1993). Each of the 41 trees had an identical substitution matrix with a total tree length of 66. As tree construction programs sometimes need to create 'steps' (i.e., backmutations) in order to build a phylogeny, the ts:tv ratio obtained from this substitution matrix is more realistic than the observed ts:tv ratio. The PAUP output (Appendix E) provides the number of steps required to construct a given phylogeny. PAUP analysis assumes that there

is no substitution bias; that each base has equal probability of occurring (unless weighting of characters is indicated).

Table 8.1: Mean numbers of transition and transversion changes at polymorphic sites among the HVR-1 haplotypes calculated using MEGA. The tv% represents the number of transversions divided by the total number of substitutions. Variances were not calculated, but are expected to be large (Wakeley, 1993).

	Mean pairwise difference			
	ts	*tv*	*ts:tv ratio*	*tv (%)*
Among dugongs	9.19	0.05	185:1	0.5
Between dugongs & the manatee	30.03	26.97	1:1	47

Regardless of whether the inferred estimate, or the observed ts:tv ratio is the more accurate, a preference for transitions among the dugong haplotypes is obvious. It is therefore appropriate to apply the Kimura two-parameter (Kimura, 1980) and/or the Tamura-Nei (Tamura & Nei, 1993) models to the data set.

8.2.2 Substitution rates among nucleotide sites

When nucleotide substitution has an equal probability at all sites (i.e., the mean is the expected substitution frequency), the data follow a Poisson distribution. The mean ($\bar{x}$) of a Poisson distribution equals the variance (s^2). If the pattern of nucleotide substitution fails to meet the 'strict randomness requirements' of the Poisson distribution (Johnson & Kotz, 1969), the variance will be significantly larger than the mean, indicating that the distribution is 'overdispersed' (Bliss & Fisher, 1953). The negative binomial distribution is the first choice of an alternative to the Poisson, and is generated when the Poisson parameter rate of nucleotide substitution (λ) varies according to a 'gamma' (type III) distribution (uneven substitution frequencies) among sites (Johnson & Kotz, 1969).

The mean (0.3402) and variance (0.5956) of the number of nucleotide substitutions per site were calculated as indicated in Schefler (1980). The

$s^2/\bar{x}$ ratio (1.7507) suggests that the distribution of nucleotide substitutions is not Poisson. A G-test (goodness of fit) employing the expected Poisson distribution was calculated as indicated in Schefler (1980) confirming this prediction (table 8.2).

The negative binomial is defined by two parameters, the arithmetic mean, $\bar{x}$, and a positive exponent, the scale parameter, k (Bliss & Fisher, 1953; the scale parameter is denoted as a by Tamura & Nei (1993), however I will continue to refer to it as k). The scale parameter can be crudely estimated by the following formula: $k = \bar{x}^2/(s^2 - \bar{x}) = 0.4532$ (Bliss & Fisher, 1953) and the expected probability frequencies (ϕ) for the number of substitution classes (x) for the negative binomial can be calculated following equations 11 and 12 from Bliss & Fisher (1953):

Equation 11 (Bliss & Fisher, 1953) for when x equals zero:

$$\phi_0 = N / q^k$$

Equation 12 (Bliss & Fisher, 1953) for when x equals 1, 2 & ≥3:

$$\phi_x = \frac{(k + x - 1).R}{x} \cdot \phi_{x-1}$$

A substitution class (x) is the number of changes occurring at a particular site (e.g., a site with two substitutions was observed at a frequency of 15 (out of 194) (table 8.2)). The expected proportions in the two contrasting categories are represented by p and q, where $p = \bar{x}/k$ and $q = 1 + p$ and $R = p/q = \bar{x}/(k + \bar{x})$ (Bliss & Fisher, 1953). The total number of nucleotides in the sequence is represented by N.

As the variance of a negative binomial approaches the mean, the overdispersion decreases and k approaches infinity and p approaches zero. Conversely, as overdispersion increases, k approaches zero. To obtain the most accurate estimate of the scale parameter (hence, expected values for the negative binomial distribution), a maximum likelihood of k was calculated using the IBM compatible program, GLIM (Crawley, 1993).

The input data for the GLIM program were the observed numbers of substitutions per site calculated from the output (e.g., Appendix E) of a parsimony analysis using PAUP (Swofford, 1993). Although 41 equally

parsimonious trees were obtained from the data (excluding the outgroup), the number of substitutions per site was the same for each (Appendix E). GLIM (Crawley, 1993) estimated the mean as 0.3402, and the variance as 0.5987. The maximum likelihood of *k* was calculated (0.3168) using the 'kfit' macro, which was then employed by the program to obtain the expected values for the G-test for the negative binomial using the 'negbin' macro.

Table 8.2: The chi squared (χ^2) or Goodness of Fit test (G-test) of substitutions per site. Observed and expected frequencies of substitutions per site, among the dugong mtDNA HVR-1 haplotypes.

Number of substitutions/site (*x*)	Hypervariable region 1 number of sites (194)		
	Observed	Expected[1]	
		Poisson	*Negative Binomial*
0	155	137	154
1	19	48	25
2	15	9	9
≥3	5	(<5)	6
		$\chi^2 = 31.4073$ (p = 0.00) 1 df.	$\chi^2 = 4.6144$ (p = 0.032) 1 df.

[1] One parameter (the mean) has been used to calculate the expected values for the Poisson distribution. After pooling expected values less than five and accounting for the one parameter, two classes remained, hence one degree of freedom (df.) exists for the Poisson comparison. Four classes were compared for the expected Negative Binomial distribution which was derived from two parameters, the mean and the scale parameter, hence one df. remains. As both expected distributions have one df., Yates correction factor was incorporated in the calculation of χ^2 in order to correct for discontinuity.

The observed distribution was significantly different from the expected Poisson and negative binomial distributions (table 8.2). Support for a negative binomial distribution in the HVR-1 exists for other species such as the southern elephant seal, *Mirounga leonina* (Slade, 1996) and humans (Kocher & Wilson, 1991; Wakeley, 1993). Given the comparatively lower probability of a Poisson distribution (table 8.2), and that the $s^2/\bar{x}$ ratio (1.7507) indicates overdispersion, I conclude that nucleotide substitution in

the dugong HVR-1 does not have equal probability at all sites, and that the method of Tamura & Nei (1993) is an appropriate model to apply to the data in the estimation of mutation rates, because it accommodates: 1) a preference in transitions over transversions, 2) a bias in substitution type, and 3) variable substitution rates among nucleotide sites (Tamura & Nei, 1993).

8.3 DIVERGENCE TIMES

Mean nucleotide distances among dugongs, and between dugongs and the Florida manatee, were obtained from the (gamma distributed) Tamura-Nei pairwise comparisons (output by MEGA, Appendix F) with a k of 0.3168. Using estimates of divergence times between the dugong and Florida manatee obtained from the fossil record (Domning, 1994) and immunological data (Rainey *et al.*, 1984), a range of substitution rates was calculated (figure 8.1 & table 8.3). These were then used to estimate the divergence times among dugong geographic units and haplotype clusters (table 8.4). The fossil divergence time is expected to be the more accurate, as explained above. Substitution rates estimated from the divergence times reported by Rainey *et al.* (1984) are included for the sake of comparison. Only divergences relevant to the interpretation of phylogeography (Chapter 11) are included in table 8.4.

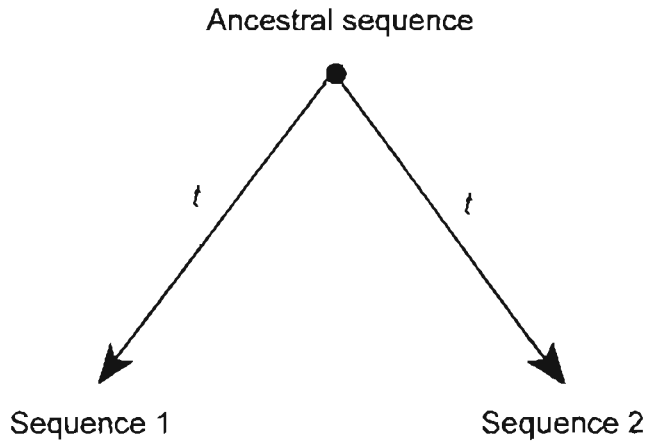

Figure 8.1: A schematic representation of how to calculate the divergence of two sequences from a common ancestral sequence *t* time units ago. The substitution rate = t x 2 lineages (substitutions per site per year).

The time since two populations (or haplotype clusters) were separated (assuming no gene flow between populations) can be estimated by the net nucleotide divergence (substitutions per site). The net divergence between the East Australian and Asian haplotype cluster was observed as 0.0858 nucleotides per site (Appendix F). Assuming a substitution rate of 2% per Myr per lineage (or 0.02 substitutions occur every Myr) based on fossil evidence (table 8.3), the divergence time for the East Australian and Asian haplotype clusters is 0.0858 ÷ 0.02 per Myr = 4.29 Myrs (divergence between 2 lineages). Thus, the East Australian and Asian haplotype clusters, are estimated to have diverged 4.29 ÷ 2 = 2.15 Myrs ago (table 8.4).

The divergences among the regions and haplotype clusters are all very similar in predicted divergence times, with the exception of the geographic units of West Indian Ocean and Australia (table 8.4).

Table 8.3: Substitution rates for the dugong. Immunological and fossil data were used as references for divergence times between the dugong and Florida manatee. Using these dates, anticipated substitution rates (substitutions/site/year) were obtained for the dugong from pairwise comparisons of haplotypes (Appendix F). Time estimates indicated in brackets are the doubled predicted divergence times to account for two lineages. The text in bold represents the most likely substitution rate.

Estimated divergence times (Myrs)		Substitutions/site/year (x 10^{-8})
Immunological data (Rainey *et al.*, 1984)		
minimum	17 (34)	3.54
maximum	20 (40)	3.01
Fossil data (Domning, 1994)		
minimum	**30 (60)**	**2.00**
maximum	40 (80)	1.50

Table 8.4: Divergence times among the dugong geographic units and haplotype clusters. The various substitution rates from table 8.3 were used to provide a range of possible estimated times since divergence between the various geographic units (section 7.3.2.3) and haplotype clusters (figure 7.4), which were obtained from average distances between haplotypes (rather than individuals). The text in bold represents divergence times with the most likely substitution rate of 2% per Myr.

Mutation Rate	Divergence time (Myr)				
	Between Regions		***Between Haplotype Clusters***		
% per Myr	Asian & all other geographic units	W.Ind- Ocean and all Australian	West Australian and Asian	West Australian and East Australian	East Australian and Asian
3.54	1.33	0.88	1.39	1.34	1.21
3.01	1.57	1.04	1.63	1.58	1.43
2.00	**2.35**	**1.56**	**2.45**	**2.38**	**2.15**
1.50	3.14	2.08	3.27	3.17	2.86

8.4 FEMALE MIGRATION RATES

As mtDNA is maternally inherited, only female migration rates were estimated. Gene flow between populations resulting from the movement of females or their gametes can be expressed as a migration rate, m. The estimate of migration rate assumes an island model of population structure where each population is of equal size N, and genes are exchanged with equal probability (Avise, 1994). These assumptions are unrealistic for most real populations, and as a result the estimates of female migration rate are probably inaccurate. Frequencies of genetic type in finite populations are also influenced by genetic drift which is a function of effective population size. It is not possible to distinguish nucleotide changes due to the effects of genetic drift from those due to gene flow (from migration), as Nm is simply the presumed absolute number of migrants exchanged between populations per generation (Avise, 1994).

According to Wright (1951), Nm can be derived from F_{ST}, the correlation of (diploid) genes of different individuals in the same population as follows:

$$N\,m \cong \frac{(1 - F_{ST})}{4\,F_{ST}}$$

Between-population heterogeneity defined by Nei (1973), namely gene diversity or G_{ST}, is a measure related to Wright's (1951) F_{ST}, and has the same relationship to Nm. Modification of the G_{ST} statistic has been suggested by Takahata & Palumbi (1985) for haploid genomes such as mtDNA. N_{ST}, an analogue of F_{ST} and G_{ST} proposed by Lynch & Crease (1990), is applicable to data at the nucleotide level (Hudson *et al.*, 1992). N_{ST} provides the ratio of the average genetic distance between genes from different populations relative to that among genes within a population (Lynch & Crease, 1990). I similarly derived this ratio from the nucleotide diversity and divergence values calculated by the program DA (REAP, McElroy *et al.*, 1992; Slade, 1996), and Nm was derived from N_{ST} (Wright, 1951; Lynch & Crease, 1990; Hudson *et al.*, 1992) as follows:

$$N_{ST} = \frac{1}{2Nm + 1}$$

$$Nm = \frac{1}{2}\left(\frac{1}{N_{ST}} - 1\right)$$

Nm values (table 8.5) are apparently an indicator of gene flow (either historic or recent) rather than active migration, and should not be interpreted as a precise estimate of genetic exchange among populations. That is, Nm values can be used to predict whether populations experience high, moderate, or greatly restricted gene flow (Avise, 1994). In theory, irrespective of population size, the average exchange of one individual per generation ($Nm \cong 1$) between populations is sufficient to prevent genetic differentiation by genetic drift alone (Allendorf, 1983). The observed Nm values (table 8.5) suggest gene flow between the nominated geographic units. Any Nm value less than 0.5 suggests very low gene flow, as observed particularly between the Asian and other geographic units (table 8.5). Higher gene flow (one or more females per generation) was suggested among the Australian geographic units and also the West Indian Ocean geographic unit. This effectively illustrates that only a few females per generation (< 1% of the population) as migrants between geographic units, are required to indicate 'high' gene flow. That is, theoretically there is a sufficient amount of gene flow to prevent genetic differentiation by genetic drift alone.

Table 8.5: Estimated migration rates (*Nm*) among geographic units. The estimated number of years for the permanent exchange of an individual female dugong between geographic units are also indicated, as calculated by the DA program (REAP, McElroy *et al.*, 1992). One dugong generation equates to 30 years. Note that the Asian (n=7) and West Indian Ocean (n=4) regions are represented by low sample sizes.

	Asian		West Indian Ocean		North & West Australian		Great Barrier Reef	
	Nm	*Years*	*Nm*	*Years*	*Nm*	*Years*	*Nm*	*Years*
West Indian Ocean	0.28	108						
North & West Australian	0.37	81	0.51	60				
Great Barrier Reef	0.48	63	0.91	33	6.17	6		
South East Queensland	0.22	135	0.49	60	0.47	63	1.07	27

8.5 EFFECTIVE FEMALE POPULATION SIZE

Estimates of effective population size, or more specifically the ratio of effective to actual (census) population size (N_e/N), can aid wildlife management by enabling predictions regarding the rate of inbreeding and loss of genetic variation (Frankham, 1995). Estimation of the effective population size (N_e) requires information from diploid (nuclear) loci, therefore this parameter could not be estimated. However, the effective female population size N_f, can be obtained from the mutation rate estimated for mtDNA, providing an estimate of the population size. However, note that Frankham (1995) found that typical estimates of effective population size (such as N_e) are about one order of magnitude less than adult population sizes. N_f can be obtained using the following formula for haploid loci such as mtDNA:

$$N_f = \frac{\theta}{2\,\mu_g}$$

Where θ, is the level of diversity per nucleotide site and μ_g represents the mutation rate per generation ($\mu_g = \mu \times g$, where μ = mutation rate per site per year, and g = generation time in years). The parameter θ is represented here by nucleotide diversity (π) (table 8.6), however θ can be estimated using other methods (Fu, 1994; Tajima, 1993).

Using the generation time of 30 years for the dugong (Marsh, 1980), and a substitution rate of 2% per Myr (table 8.3), effective female population sizes for each group were calculated and the estimates are presented in table 8.6.

Table 8.6: Estimated effective female population size and % nucleotide diversity (π) within each geographic unit. Within-geographic unit nucleotide diversities with a Tamura-Nei correction (k = 0.3168), were calculated from pairwise comparisons of dugong haplotypes using MEGA . Note that the estimates of effective female population size are likely to be imprecise.

Within geographic unit	*% Nucleotide diversity (π)*	*Effective female population size*
Asian (n=7)	6.33	34,750
West Indian Ocean (n=4)	5.09	46,583
North & West Australian (n=29)	4.17	29,912
Great Barrier Reef (n=37)	5.59	52,750
South East Queensland (n=26)	3.59	42,417
	Average 4.95	*Total* 206,412

8.6 DISCUSSION

8.6.1 Substitution rates & divergence times

The observed frequency of transversions among dugong haplotypes was very low at 0.5% of all substitutions (table 8.1). This is an unusually low amount of transversions compared to the 5% observed in the HVR-1 of humans (Wakeley, 1993) and 4% for the southern elephant seal (Slade, 1996). The preference for transitions over transversions, in addition to the uneven and biased substitution type among nucleotides of the HVR-1, indicated that the method of Tamura & Nei (1993) was the most appropriate model to estimate distances between pairwise comparisons of haplotypes. However, even with the Tamura-Nei correction, Perna & Kocher (1995) predict that the subsequent mutation rates obtained from the HVR-1 of

mtDNA will be underestimated. Perna & Kocher (1995) argue that because the Tamura-Nei model uses the average composition of the sequence as an estimate of the equilibrium nucleotide frequency, substitution inequalities such as a bias against the nucleotide guanine, will also be underestimated.

Substitutions in the HVR-1 of mtDNA do not fit a Poisson distribution. The borderline rejection of the negative binomial indicates that it is the better fit for describing the nature of substitutions in the HVR-1 of mtDNA in the dugong, as confirmed for other species (Kocher & Wilson, 1991; Wakeley, 1993; Slade, 1996).

The scale parameter obtained (k = 0.3168) for the dugong data was most similar to that observed in humans for the HVR-1 (k = 0.47, Wakeley, 1993), although it is slightly closer to zero (i.e., more overdispersed). The scale parameter (k) is expected to be slightly overestimated for the negative binomial distribution, because not all backmutations are accounted for in phylogeny construction, and the number of substitutions per site (mean and variance) are underestimated (Wakeley, 1993). As a consequence, lower than actual estimates of distance among haplotypes are obtained, and predictions of divergence times (from the estimated substitution rates) are conservative.

Substitution rates are five to ten times higher in the mtDNA genome in comparison to protein-coding nuclear DNA. However, some species such as *Drosophila*, vary little in substitution rates between the nuclear and mitochondrial genomes (table 8.7). The mean rate of nucleotide substitution/divergence averaged over the entire mtDNA molecule can be as low as 0.13% per Myr for echinoids (table 8.7) and average 2% per Myr for most vertebrate species (table 8.7). Substitution rates are generally higher in the control region (D-loop) compared to the rest of the genome (table 8.7). The D-loop region of mtDNA is expected to have a higher rate of evolution than protein coding regions, as it is not under the same evolutionary constraints. In addition, HVR-1 and HVR-2 (5' and 3' of the central conserved domain respectively, figure 6.1) of the D-loop have especially high rates of evolution.

Predicted substitution rates for HVR-1 are 3% to 3.5% based on immunological data, and between 1.5% and 2% based on fossil data (table 8.3; Rainey *et al.*, 1984; Domning, 1994). The divergence times estimated by

Rainey *et al.* (1984) for sirenians are probably underestimated, because of the faster rate of evolution suggested for the elephant (Irwin & Arnason, 1994; Lavergne *et al.*, 1996; Ozawa *et al.*, 1997). The most accurate estimate of substitution rates should be gained from sirenian fossil evidence (table 8.3; Domning, 1994). Therefore, I use 2% per Myr as the rate of mutation in the dugong's HVR-1 of mtDNA (table 8.7). This is a lower estimate than the rate observed for this region of other species, although somewhat comparable to the rate observed for the horse (table 8.7; Ishida *et al.*, 1995). For most species, the rate of about 2% per Myr appears to be the norm for the mtDNA molecule as a whole (table 8.7), rather than a rate for the control region in particular.

Estimating substitution rates (table 8.3) permitted estimates of divergence times between Asian, West Indian Ocean and Australian dugongs (table 8.4). How these divergence dates relate to historical geographical events creating possible gene flow barriers is covered in Chapter 11.

8.6.2 Female migration rates

Under the neutrality theory, at equilibrium, the level of divergence among populations is a function of the absolute number of migrants rather than the proportions of individuals exchanged. This procedure of deriving Nm should *not* be interpreted as providing a precise estimate of genetic exchange among populations. It simply offers a qualitative guideline for the extent of presumed gene flow (Avise, 1994). As the estimate of Nm is entirely dependent on the estimate of N_{ST}, it will be directly influenced by sample size. Therefore, estimates of female migration between dugong populations represented by small sample sizes (less than five, see section 3.9) are unlikely to be accurate. In addition, the assumption of equilibrium is rarely if ever met in real populations. However, Nm is still effective in illustrating the assumed proportion of (historical or recent) gene flow that may have occurred between populations. It may also be predicted that because of the high connectivity (openness) of the marine environment, the Nm of a marine species will be relatively higher than terrestrial species.

Table 8.7: Substitution rates estimated for the entire mtDNA molecule, entire control region, and control region I (i.e., HVR-1). Assuming a date from sirenian fossil data (Domning, 1994), a HVR-1 substitution rate was predicted for the dugong. This table was adapted from Moritz *et al* (1987) and Wilson *et al.* (1985).

Substitution rate (% per Myr)	*Species*	*Reference*
control/hypervariable region 1		
1.5 - 2	**dugong**	**this study**
10.3	human	Horai *et al.* (1995)
2 - 4	horse	Ishida *et al.* (1995)
7.5	southern elephant seal	Slade (1996)
entire control region		
7.5 [b]	human & chimpanzee	Tamura & Nel (1993)
21.3 [b]	"	Perna & Kocher (1995)
11.4 - 11.8	human	Stoneking *et al.* (1992)
7	human	Horai *et al.* (1995)
0.3 - 1	cetaceans	Hoelzel *et al.* (1991)
mtDNA molecule		
2	primates	Brown *et al.* (1979)
"	"	Brown *et al.* (1982)
2.5	"	Hasegawa *et al.* (1985)
0.5 - 1 [a]	"	Hixson & Brown (1986)
0.5 - 1 [a]	"	Vawter & Brown (1986)
2	*Chelonia mydas*	Encalada *et al.* (1996)
0.13 - 1.1	echinoids	"
0.6 - 1.8 [a]	*Drosophila*	Powell *et al.* (1986)
≈ 5 [a]	"	DeSalle *et al.* (1986)
2 - 4	*Mus*	Ferris *et al.* (1983)

[a] As cited by table 1 in Moritz *et al.* (1987).
[b] Using the same data set for the human and chimpanzee.

Migration of an individual every one to two generations between the West Indian Ocean and Australia is suggested from the estimates of $N\,m$ (table 8.5), but highly unlikely because of the considerable geographical separation of these populations (see Chapter 11 for discussion on this). The

supposed migrations are probably reflecting the genetic similarity of these geographic units. West Indian Ocean is represented by a small sample size (only four individuals, table 7.2) which will also reduce the accuracy of the *Nm* estimate. Simulations by Slatkin & Barton (1989) showed that θ over-estimated *Nm* when sample size was small. With respect to Australian populations, the suggested migration of one female every one to two generations among geographic units (table 8.5) is believable considering the geographical closeness of the populations, and is supported by the regional overlap of haplotypes. The *Nm* values suggest that Asia is the most isolated dugong geographic unit, which is probably better explained by the high genetic diversity and small sample size, rather than its phylogenetic relationship to other haplotypes (figure 7.4). The migration rate also suggests that dugongs of the Great Barrier Reef and Asia are the most closely related compared to the other geographic units. The probability of movement of female dugongs from Asia to the east coast is discussed in Chapter 11.

8.6.3 Effective female population size

Aerial survey estimates of dugong numbers in Australian waters and elsewhere in their range (tables 2.3 & 2.4 respectively; section 2.4.4) barely approximate the effective female geographic unit sizes listed in table 8.6, with the exception perhaps of the North & West Australian geographic unit. If effective female geographic unit sizes were doubled to include male dugongs (the dugong male:female birth ratio is 1:1, section 2.4.5), the discrepancy is further increased. Population estimates from aerial surveys are generally underestimated (H.D. Marsh, personal communication). However, both aerial survey and genetic methods for estimating population size are inaccurate, which is expected to further bias *Nf* values relative to census population size. Frankham (1995) attributes variation in estimates of the ratio between effective population size and actual population size to (in order of importance): 1) fluctuation in population size, 2) variance in family size, 3) form of *N* used (e.g., adult versus breeders verses total population size), 4) taxonomic group, and 5) unequal sex ratio. In addition, the estimates from genetic data are probably imprecise because of the lack of definition of precise boundaries of the geographic units, and the substantial level (in genetic terms) of presumed gene flow. That is, the *Nf* values may be relevant for a genetic neighbourhood that is much larger than the geographic units defined in this study.

A further effect on the estimate of *Nf* values is the large variance associated with nucleotide diversity. Estimation of the effective female population size is dependent on the measure of nucleotide diversity. The average number of nucleotide differences (nucleotide diversity) is typically accompanied by a large variance (Tajima, 1983), particularly when the time since divergence between two populations is relatively short. In addition to recent migration between populations, or short divergence time among populations, as an explanation for the (large variance) relationship, Tajima (1983) included natural selection (conservation of particular haplotypes) and stochastic error generated by random genetic drift. The calculation of nucleotide diversity is dependent on sample size, which may also add to the variance (Tajima, 1983). However, because of the large variance of nucleotide diversity, increasing the sample size will not reduce the variance. A sample size of five to ten individuals is nearly as reliable as a sample size of 200 haplotypes to represent within-population genetic diversity (Tajima, 1983). In summary, small sample sizes are less likely to be the source of the disparity (of several thousand individuals) in the genetic estimation of population size. A more likely explanation is provided by the large variance associated with measures of nucleotide diversity. Any factors that affect nucleotide diversity (e.g., bottleneck) will in turn affect making an accurate estimate of population size. In conclusion, despite the inaccuracies associated with aerial survey and genetic approaches to estimate population size, both have supported the notion that the total number of dugongs in Australian waters is large, that is, over 100,000 animals.

8.7 CONCLUSIONS

The Tamura-Nei model (Tamura & Nei, 1993) accommodates differing rates of transitions to transversions, and unequal among-site substitution rates. For these reasons, it was deemed the most suitable model to correct the data obtained from the HVR-1 of mtDNA. Using the corrected distance matrix, a substitution rate of 2% per Myr has been estimated. This substitution rate is within the range of rates observed for other vertebrate species.

The Tamura-Nei model (Tamura & Nei, 1993) is the more realistic and the more accurate than Kimura's two-parameter model (Kimura, 1980), in calculation of genetic distance. However, estimates in the number of substitutions by Tamura-Nei are associated with a much larger variance.

This larger variance is partly due to the introduced scale parameter. Further contribution to the large variance, particularly with small sequences (e.g., 20 nucleotides), is provided by the logarithms used to estimate distance (Tajima, 1993). However, as the HVR–1 sequences are relatively large (194 nucleotides), this effect is assumed to be negligible. Parsimony analysis is another source of error as it underestimates the actual number of substitutions per site and as a result, divergence times will be conservative.

From the mutation rates, *Nm* values were estimated, suggesting the degree of gene flow (historical or recent) between the geographic units was high (one or more individuals per generation suggests high gene flow). Divergence times estimated (from fossil data) between the Asian and West Indian Ocean, and Australian dugong geographic units coincide with the end of the Pliocene for a common ancestor.

CHAPTER NINE

MICROSATELLITE LOCI

This chapter expands on the introduction presented in section 2.2.5, by discussing why microsatellite markers were chosen to investigate the intraspecific genetic structure of the dugong. A number of microsatellite loci were located from a dugong genome library. Five of these were sequenced and two were trialed; the microsatellite located in the control region of mtDNA in the dugong (Chapter 6), and one of the five microsatellites from the genome library. Unfortunately I was unable to show whether the microsatellites were informative in relation to the genetic population structure of the dugong. However, a respectable foundation for future research in this area has been laid. The application of microsatellites to examine intraspecific differentiation in other species has been reviewed and is discussed.

9.1 WHY USE MICROSATELLITES IN PREFERENCE TO OTHER GENETIC MARKERS?

Microsatellite loci are currently fashionable as genetic markers for intraspecific studies. The application of microsatellites is popular whenever Mendelian markers are required, in areas as diverse as: forensics (Budowle *et al.*, 1991; Roewer *et al.*, 1991), clone or strain identification (Brookfield, 1992), systematics (Schlötterer *et al.*, 1991), genetic mapping (Hearne *et al.*, 1992; Weissenbach *et al.*, 1992) and familial investigations (as discussed by Queller *et al.*, 1993).

Microsatellites exhibit the following useful features: 1) the information obtained is consistently scorable and comparable (section 9.1.1), 2) they are polymorphic with relatively low mutation rates (section 9.1.2) and, 3) they are neutral (9.1.3) (Queller *et al.*, 1993). An additional advantage of using microsatellites, is that the alleles can be amplified via PCR (as introduced in section 4.3).

9.1.1 Consistently scorable and comparable

Microsatellites, despite their polymorphism (i.e., multiple alleles), are easily and consistently scored without confusion (Queller *et al.*, 1993). This is made possible by employing the PCR which can amplify microsatellites to particular

sized fragments, targeted to run in distinct areas of a gel (Queller *et al.*, 1993; Tautz, 1989; Weber & May, 1989).

A concern with employing multi-locus methods such as DNA fingerprints and RAPDs (section 2.2), is the lack of repeatability. The comparison and interpretation of gels run at different times can be difficult. Even if the bands have been consistently scored, they are not truly informative unless they accurately map their underlying genes (Queller *et al.*, 1993). Microsatellites can be accurately compared across gels because their bands normally differ by uniform, known and resolvable distances. In practice, the pattern given by microsatellites can be slightly complicated, with each allele showing 'stutter bands' (a ladder of bands of nearly equal intensity). Stutter bands are thought to be due to the DNA polymerase slipping during PCR. To circumvent this problem, use of trinucleotide repeats is preferred, as they are less likely to produce stutter bands than dinucleotide repeats.

9.1.2 High polymorphism and relatively low mutation rates

Molecular markers with high variability are desirable for intraspecific studies because of the resolution they can provide. For example, Spencer *et al.* (1997) reported an average heterozygosity in a colony of rock-wallabies (*Petrogale assimilis*) of 85.5% using microsatellites, compared to 3.8% using allozymes, or 47.3% from multilocus 'DNA fingerprints'.

Unfortunately, high mutation rates often accompany high variability (Weissenbach *et al.*, 1992), and can be a problem, as they will cause a marker to provide misleading information about the genetic history of an individual (Queller & Goodnight, 1989; Queller *et al.*, 1993). Microsatellites generally provide a satisfactory compromise; they have high polymorphism and mutation rates typically below 10^{-4} (length variant/motif per gamete per kilobase) (Weber, 1990; Edwards *et al.*, 1991; Edwards *et al.*, 1992; Hearne *et al.*, 1992; Weissenbach *et al.*, 1992). Minisatellites (DNA fingerprints and VNTRs) which are popular in their application in forensics and as a medical tool (because of their individual specificity) have mutation rates as high as 5×10^{-2} (length variant/motif per kilobase per gamete) (Jeffreys *et al.*, 1985a). However, because of their extremely high variability, minisatellites are generally less suitable as a genetic marker for a population level study.

9.1.3 Neutral

A genetic marker under strong selection, is not a good marker for anything except itself (Queller *et al.*, 1993). DNA markers, especially from non coding regions such as the D-loop of mtDNA, minisatellites and microsatellites, are expected to be more neutral than allozymes. Although some microsatellites are known to experience selection, as they have been identified as responsible for genetic defects (Hearne *et al.*, 1992; Richards & Sutherland, 1992), most are considered neutral.

It is desirable to employ at least ten microsatellite loci in a population structure study. This enables the researcher to detect loci that are behaving unexpectedly and may bias results. Any microsatellite locus behaving as an outlier can be tested for associative selection pressure (hitchhiking and overdominance) as shown by Slatkin (1995).

9.1.4 Interpretation of microsatellites

Simple or uninterrupted repeats (figure 9.1) are the easiest to interpret; there is either a PCR product representing an allele of a particular length, or there is not. When interrupted or compound repeats (figure 9.1) are used, the interpretation becomes more complicated as the alleles in each sample differ not only in length, but also in sequence.

The interpretation of microsatellites is much the same as for allozyme data where the frequency of each allele in a population is examined. Genetic diversity (polymorphism) can be measured as the mean number of alleles per locus, observed heterozygosity and that expected from Hardy-Weinberg assumptions (Nei, 1987). The Fisher's exact test (Sokal & Rohlf, 1981), Nei's unbiased genetic distance (Nei, 1978), analogues of Wright's *F*-statistics (Weir & Cockerham, 1984), and the log-likelihood ratio test (G-test of independence) (Sokal & Rohlf, 1981), can all be used to compare the level of variation among populations (heterogeneity). Software programs available to assist in these statistical interpretations include: BIOSYS-1 (Swofford & Selander, 1981), GENEPOP (Raymond & Rousset, in press) and FSTAT (Goudet, in press).

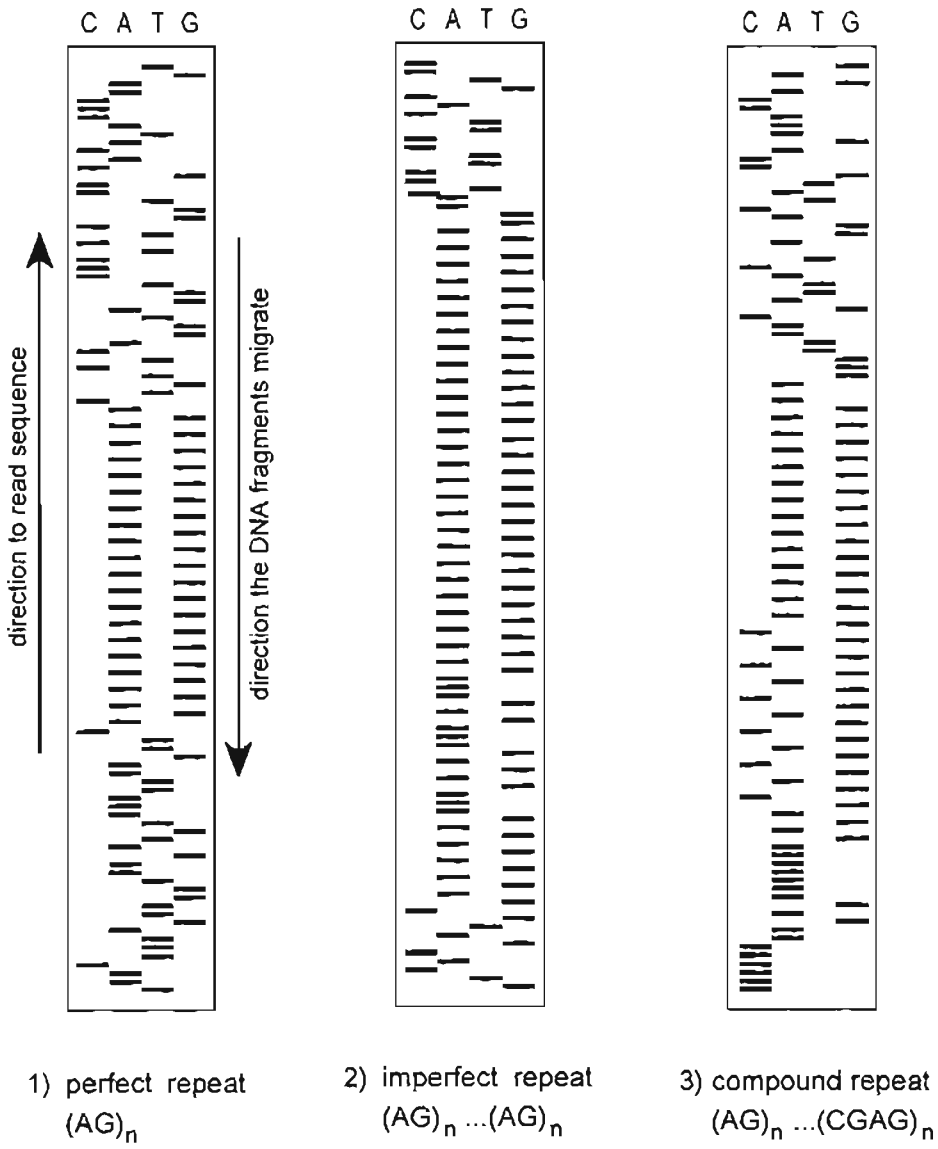

Figure 9.1: A schematic representation of the three classes of microsatellites, as would be visualised on an autoradiograph. 1) The perfect repeat, which is a long and continuous stretch of the repeating unit, 2) the imperfect repeat which is interrupted by miscellaneous bases, and 3) the compound repeat which consists of adjacent arrays of different repeat types(A. Brooker, personal communication).

9.2 APPLICATION OF MICROSATELLITES

9.2.1 Microsatellites in population structure studies

As microsatellites generally exhibit high levels of polymorphism, they can sometimes reveal intraspecific genetic variation and differentiation when other genetic markers have failed. They are particularly relevant for studies of species with a low effective population size, which are typically characterised by low levels of genetic variation due to low population densities. For example, both mtDNA analysis and multilocus DNA fingerprints failed to reveal population differentiation in the northern hairy-nosed wombat (*Lasiorhinus krefftii*) (Taylor *et al.*, 1994). In contrast, microsatellite loci enabled estimates of heterozygosity which showed a severe loss of genetic variation in the Epping forest colony of this species consistent with its 120 year decline (Taylor *et al.*, 1994).

Pope *et al.* (1996) used both control region mtDNA sequences and microsatellite loci to differentiate Queensland and South Australian yellow footed rock wallabies (*Petrogale xanthopus*). Distant populations (70 km apart) of the wallaby were distinguished with the mtDNA sequences and microsatellites. However, close populations (10 km) were distinguished only by microsatellite loci. The genetic results were consistent with ecological studies, which suggested that dispersal between colonies was limited. Hence for management purposes, each colony needed to be considered as an independent unit.

Microsatellite loci were able to provide measures of genetic variation among Canadian black bear (*Ursus maritimus*) populations, when allozyme and mtDNA analysis showed little or none (Paetkau & Strobeck, 1994). Three Canadian black bear populations had relatively high levels of heterozygosity (average 80%), whereas the Island of Newfoundland population had a low level (average 36%). Heterozygosity of Canadian polar bears averaged 60% within each population (Paetkau *et al.*, 1995), and measures of genetic distance between the populations suggested a pattern of gene flow reflecting movements of individuals not obvious from their geography.

9.2.2 Social structure

Because of their high polymorphism, microsatellite loci are an ideal genetic marker to examine the social structure in a species of interest. For example, Amos *et al.* (1993) used a combination of one minisatellite locus and six microsatellite loci, to resolve the social structure of Faroese long-finned pilot whales (*Globicephala melas*). A correlation between the age of a female pilot whale, and the probability of observing its genotype in a pod (Amos *et al.*, 1993), suggests more mother-offspring than father-offspring relationships in a pod (Hoelzel, 1994). The conclusion was that pilot whale pods are matrifocal with males remaining with their natal group, but dispersing to other pods to mate (perhaps during interactions between pods).

9.3 MICROSATELLITES AND POPULATION DIFFERENTIATION FOR THE DUGONG

Highly variable, easy to use, abundant and providing unambiguous genetic data, with information limits set by time and money rather than biology, microsatellites appear to be the unequivocal choice of genetic marker for fine scale population resolution (Queller *et al.*, 1993). However, a genome library needs to be constructed in order to find microsatellites (section 4.6). Construction of a genome library is tedious and time consuming. Furthermore, many microsatellite loci must be screened before loci with sufficient flanking sequences for primer design and population-specific size ranges are found.

The construction of a genome library can be bypassed if primers from closely related species are available. Often the same primers can be used among closely related species to amplify the same microsatellite loci. Some cow primers work with sheep (Moore *et al.*, 1991) and common primers have been applied to a range of cetaceans (Schlötterer *et al.*, 1991). Using recognised microsatellite primers of a closely related species would save much time and cost in the laboratory. As no microsatellite primers had been published for sirenians or closely related orders (i.e., proboscideans, hyracoids or tubulidentates), I chose to search for microsatellites in the dugong as detailed in section 4.6 and summarised in figure 4.4.

```
F1C38   10          20          30          40          50          60
CTCGGTACCT CTTTCAGTTG GTTTCTGTCC CTTTAACATG CTAGTCTTTG TGTGTGTGTG
TGTGTCTGTG TCTGTGTGTG TGTGCATGCG TGTGTGTGTG TTTATTTAGC ACTTTTTTAC
TTTCTGGCAT GACAAGATAT TCCATGTTCA TATTGTGTAT TTCCTGCCTC AGTCCTAGAA
TTAGCCTTTT CTCCAGAAGC CATGGTTCCT TTTATTGGAA GATGGTGTTA GAAAAAAAAG
TATCTGGCAA TTGGTT

F1C46   10          20          30          40          50          60
AATTTAATAC GCTCTCTGTA TGTTTTTAAG AAATAGCACC CATTTTGCCG TCTCCGGTTT
AGCGGCTAAA TGATTTAAGT GTTGGAAATG TCGCTCTGAG GATGGAGGGT AACCAGTTCA
GAGAAATCTG GGAGGCTGGG GTXGGGGTGG TGGGTTCAGA GCCAGCCAGC AGCATCTTGG
CCTGGGTGTG AGTGTGCATG CATGTGTGTG TGTGTGCATG TGGTACCGAG CTCGAATTGC
TAATCATGGT CATAGTGCTT TTGTGTGAAA TGTTATCGCT CACAATTCAC ACACATACGA
GCCGAAGCAT AAAGTGT

F2C5    10          20          30          40          50          60
GGAGGGGATG AAGTCGTAAA GTGTGTGTGT ATGTGTGTCC AGGGCTCCTT CTTCCACCTC
TAGCCGTCTT GCTTTCTGTC CTGTGTGCGT TTGGGGGGCG GCCAAGGGAG AGTAGGTGGT
GGGGCTGGGG AGGGGGATGG GCTGCCGCTT TGTTCGCCTC CTTCTTGCCT TCCCTGCCGA
GGAGGATAGG TACCGAGCTC

F2C73   10          20          30          40          50          60
AAAAGGTCAG CAGTTGCAAT CCACCATCCA CTCCTTGGAA ACTCTGTGGA GCAGTTCTGC
TCTGTCCTAT AGAGTCGCTA TGAGTTGGAA TAGACTTGAT GGCAGCGTGT TTTTTTTTTT
GTTTTTTTTT TTTGTGTGTG TGTGTGTGTA AATACGAGTG ATAATTCTAT CTCTGGAGCC
CTGGTGGCAC AGTGGTTAAG AGCTGGACTG CTA

A2C70   10          20          30          40          50          60
CTAGCCCTGA CTGAAGAATA AACCCTCTGT GGCTGCGGAT CACGGACGGG ATGCCTAGGG
TTTGAACTGG ACTGGAGATG TGTTGCTGCC TCAGAAAATC TAACCACACA TTGAAATAGA
GAGGAGGTGG AATAAATAAA ATGTGTGTGA GTGTGTGACA GTGTATATGC ATGTAAGGTG
TGTGTGTGTG TGTGTGCGTG TGCTGCATTT GCATGTGAGT GTGTGTGTGT GTGTGTGTGT
GTCATGTTAG TGAATGTTGC ATGTGAT
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Figure 9.2: Sequenced microsatellites from the dugong genome library. Of those clones that screened positive with a $(GT)_{10}$ oligonucleotide probe, five revealed imperfect microsatellite repeats when sequenced. Numbers at the top of each sequence, are arbitrary nucleotide positions. At position 143 in clone F1C46, the letter X indicates a cross band. The repeats are underlined. Of the five clones positive for microsatellite sequences, one primer pair, italicised in the F2C73 sequence (i.e., MS1 and MS2, table 4.1), was designed from the flanking regions to the microsatellite following guidelines in section 4.3.1.

9.3.1 Methods and Results

Approximately 1700 positive clones (for plasmid-insert) were picked and fixed to filters (genome library), of which 31 produced a signal when screened with the following radioactive labelled probes: $(GATA)_7$, $(CAC)_{10}$ $(CGG)_{10}$, and $(GT)_{10}$. All of the 31 clones were sequenced. Five that were positive when

screened with the $(GT)_{10}$ probe, revealed microsatellites with substantial flanking sequences suitable for designing primers (figure 9.2). Of the five sequenced microsatellite loci, one primer pair (MS1 & MS2) was designed (section 4.3.1) and manufactured to amplify the GT-AC compound microsatellite (clone F2C73) indicated in figure 9.2.

The utility of the PCR products amplified by these primers to show intraspecific subdivision, was assessed along with the repeat region (microsatellite) of the D–loop introduced in Chapter 6 (amplified by the primer pair, A78 & A80). All the microsatellite primer sequences are listed in table 4.1. DNA from five Moreton Bay and five Torres Strait dugongs was amplified following section 4.3.3 and rerun as 'hot PCR' (section 4.6.11) on an acrylamide gel. These experiments were done twice. In both instances, bands had been seen on an agarose check gel after PCR. When the MS1 and MS2 products were run on an acrylamide gel, no bands were visible. With respect to PCR products amplified with A78 and A80, a series of bands were visible in the correct size range. However they were too poor in quality for interpretation and were thought to be background (i.e., PCR artefacts). It was likely that the PCR of these microsatellites required further optimisation (N. Fitzsimmons, personal communication), but I was unable to carry out further trials due to time constraints.

9.3.2 Comments and Future Research

It is evident from this study that microsatellites exist in the dugong genome. I took six months to work up a gene library, adequately sequence five microsatellites (in addition to the repeat in the control region) and trial two. I estimate that to achieve the aim of using microsatellites to recognise intraspecific genetic structure for the dugong, would take another three months to two years. All the clones positive for microsatellites have been stored as glycerol stocks (at -70°C in the DNA laboratory, Department of Zoology and Tropical Ecology, James Cook University of North Queensland), therefore a substantial foundation for the development of these markers in a future study has been established. The dugong primer sequences I developed have been sent to a research group in Florida (laboratory of Peter McGuire & Brian Bowen) to investigate if they are informative (i.e., population specific) in manatees.

CHAPTER TEN

HYPERVARIABLE REGION 1 DNA SEQUENCE: APPLICATION TO WILDLIFE FORENSICS

Dugongs are protected within Australian waters with the exception of traditional hunting by Aborigines and Torres Strait Islanders. The export and import of dugong material for research is regulated by CITES under Appendix II of the *Australian Wildlife Protection Act 1982*. In situations when protective legislation is possibly violated and confirmation of the biological material as dugong for law enforcement is required, DNA analysis is a reliable and definitive confirmatory tool. This chapter details the use of DNA sequence information of the hypervariable region 1 (HVR-1) of mtDNA to: 1) identify biological material as dugong, 2) indicate the minimum number of individuals, and 3) suggest the geographic origin of the specimen(s).

10.1 PCR and WILDLIFE LAW ENFORCEMENT

The exploitation and poaching of protected species such as the dugong is of concern to conservationists. Dugong exploitation through illegal hunting is also of concern to those Indigenous communities for which the dugong is an important food resource, central to their culture, economy and religion (Chase, 1981; Johannes, 1981; Johannes & MacFarlane, 1991).

Enforcing protection from illegal hunting is a problem throughout the dugong's range (Marsh & Lefebvre, 1994). Wildlife law enforcement requires accurate identification of the biological material of interest, which may be, for example, a 'skinless piece of meat'. DNA analysis is an appropriate and definitive approach to identify the species of origin.

The Polymerase Chain Reaction (PCR, section 4.3) aids DNA analysis in a way that has revolutionised forensic investigations. DNA can be extracted and amplified from a wide range of tissues including: hair roots (Higuchi *et*

al., 1988; Vigilant *et al.*, 1989), bone (Hagelburg *et al.*, 1991a; 1991b; Hagelburg & Clegg, 1993), faeces (Tikel *et al.*, 1996), fingernails (Kaneshige *et al.*, 1992), saliva and semen (Roewer *et al.*, 1991).

A well publicised example of the application of DNA analysis in wildlife law enforcement was the detection of illegally hunted whale products available on the Japanese market (Baker & Palumbi, 1994; Baker *et al.*, 1996). Despite a moratorium on commercial hunting in 1986 by the International Whaling Commission (IWC), some IWC members continue to hunt whales under scientific permit and as a result, the commercial market for whale products has been maintained (Baker & Palumbi, 1994; Baker *et al.*, 1996). Baker & Palumbi (1994) indicated how scientific whaling served as a cover for the illegal sale of whale products in Japan. The meat was dried and/or salted, marinated or raw. In order to comply with restrictions on the importation and exportation of whale products for scientific research, DNA was amplified via PCR from whale meat in Japan (Baker & Palumbi, 1994). After PCR, DNA is regarded as 'artificial' rather than 'whale', hence the PCR products could be exported legally and sequenced in a laboratory outside of Japan. Several protected whale species were identified in addition to those hunted and traded in accordance with international treaties (Baker & Palumbi, 1994; Szabo, 1994; Baker *et al.*, 1996), demonstrating a need for systematic genetic testing of commercial whale products.

In this chapter, I present two case studies in which the HVR-1 of mtDNA was amplified and sequenced to identify biological material as dugong. The information I provided was, in the end, not required for the court decision in either case. However, I have illustrated the ability and reliability of DNA sequencing of the HVR-1 of mtDNA to: 1) determine whether a sample is dugong, 2) indicate the minimum number of individuals present among a collection of samples, and 3) suggest the geographic origin of each sample.

10.2 CASE STUDY 1: ILLEGAL REMOVAL OF DUGONG MEAT FROM LOCKHART RIVER COMMUNITY

10.2.1 Introduction

Fourteen pieces of suspected dugong meat (composed of skin, fat and muscle) were seized in Weipa (12°41'S, 141°53'E; figure 10.1) in November 1993 because of the alleged illegal removal of the meat from the Lockhart

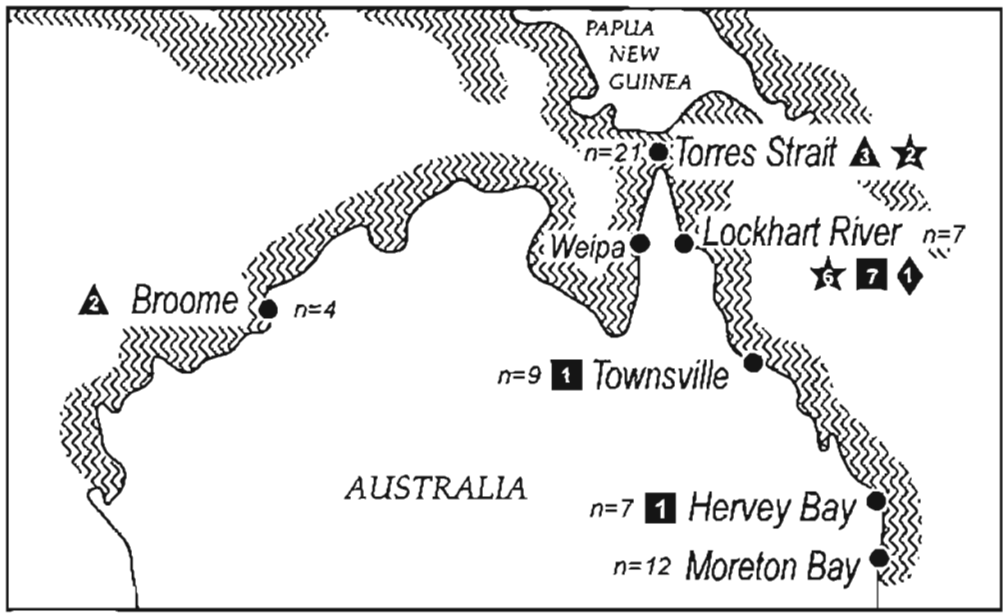

Figure 10.1: Frequency of haplotypes observed in *Case Study 1 and* 2. The frequency of each haplotype as detailed in table 7.2, are indicated within the corresponding symbols. Haplotype numbers refer to the sequences listed in table 7.1. The total number of samples collected from each locality is indicated by *n*. The three haplotypes observed in *Case Study 1* occur in the Torres Strait, Townsville and Hervey Bay dugong populations. The haplotype observed in *Case Study 2* (haplotype #3) was also observed in a Torres Strait and Broome dugong populations.

River Community (12°58'S, 143°31'E; figure 10.1) (Deirings, 1993). Dugong meat is a protected marine product under Section 45AA(1) of the *Queensland Fishing Industry Organisation and Marketing Act 1982*. As the defendant took a protected marine product for his own consumption away from the community in which he was allowed to consume it without authorisation, he was charged accordingly (Deirings, 1993).

Biologists at James Cook University of North Queensland, Townsville (19°16'S, 146°49'E), were asked to confirm if the meat was dugong. The skin of dugong is distinctive (Bryden *et al.*, 1978). Grey coloured, with fine hairs scattered over the entire body, dugong skin has no sweat glands and their 'blubber' (insulating layer of fat) is much less developed than in most other marine mammals (Domning, 1977; Bryden *et al.*, 1978). The grey epidermal layer is about a millimetre thick. Inferior to the grey layer, is a white fibrous hypodermis which can be up to several centimetres thick (see figure 3.5). The muscle of the dugong is light coloured (i.e., more like pork than beef) and, if observed independently of the skin, is difficult to verify as dugong.

The HVR-1 sequence of mtDNA of sirenians is characteristic of the order. The Florida manatee, a close relative to the dugong, differs from the latter at approximately 30% of the sites in this region (figure 6.2). Despite the high variability of HVR-1, this segment of DNA is not specific to individual dugongs. Thus only a minimum (rather than an absolute) number of individuals can be estimated from a group of specimens.

10.2.2 Methods

The 14 samples were frozen when they arrived (January 1994) at James Cook University, although considering the remoteness of Lockhart River, it is likely that the samples had been defrosted more than once during transit. The samples were thawed and the DNA extracted (section 4.1). The quality and quantity of DNA was checked (section 4.2). In total, I obtained approximately 2 µg of high molecular weight DNA from about one gram of tissue (muscle and skin). Some of the samples were partly degraded, but all had some high molecular weight DNA. Up to 20 ng of the genomic DNA was used as template for the PCR (section 4.3). HVR-1 in the dugong was specifically amplified by the mtDNA control region forward primer, L15926 (Kocher *et al.*, 1989) and dugong-specific reverse primer, A58 (table 4.1). The PCR products were either sequenced directly, or cloned and then sequenced

(section 4.4). In addition, skin, muscle and liver from an individual dugong were sequenced to check if site heteroplasmy (refer to section 2.2.10) had occurred.

10.2.3 Results and Discussion

The pieces of skin were initially identified by visual inspection as dugong based on their macroscopic appearance (Domning, 1977; Bryden *et al.*, 1978), and a statutory declaration to this effect was signed by dugong biologists: H. Marsh, G. Heinsohn & A. Preen (Deirings, 1993). Subsequently, the HVR–1 of mtDNA of all 14 samples were sequenced and all were confirmed as dugong by comparative alignment to other known dugong sequences and a closely related outgroup, the Florida manatee (figure 10.2). The level of genetic variation was 1% to 7% among the dugong haplotypes (H) (compared to approximately 30% between the dugong and the Florida manatee) (figure 10.2).

Among the 14 pieces of tissue, three haplotypes were observed (#10, #11 and #13 as shown in figure 10.2). All of the haplotypes are known from Australian dugong populations only (Chapter 7). Seven of the 14 pieces of meat were H#10, a haplotype which has also been observed in Torres Strait dugong populations (figure 10.1). Six of the 14 were H#11, a common haplotype which has also been observed in dugong populations of Townsville and Hervey Bay (figure 10.1). Haplotype #13 was observed in only one of the 14 tissue samples, and is an unique haplotype (figure 10.1).

A check for site heteroplasmy in the HVR-1 of various tissues of one dugong (i.e., muscle, skin and liver of one individual) proved negative, suggesting that more than one haplotype is unlikely from the same individual (although tissues from more individuals need to be tested to substantiate this conclusion). This suggests a minimum of three individual dugongs were represented by the 14 pieces of tissue. The defendant had indicated that the 14 bags of meat were from one individual. My findings refute this claim.

The bags of meat were identified beyond doubt to be that of the dugong. Although the defendant was charged with the illegal removal of dugong meat from the community where he was allowed to consume it, he was subsequently 'discharged absolutely without conviction' pursuant to

```
TS dugong   CCAGTACGGTAGGATTCATGCTCTAAAGCCTAAGTAATTAATCTCCATTATACAACCTCT 60
MB dugong   .......A.........................A...CC..CT....C..........C 60
CS1  H#10   .......A.........................GA...CC..CT....C..........C 60
CS1  H#11   .......A.....................................................C 60
CS1  H#13   ..........................................................  60
CS2  H#3    ..........................................................  60
Fl manatee .A.....A...T..G.A......CT.GA.AG---..TA..CCT....C.GC.G.TT.C.A 57

TS dugong   ACACCATGGATATTGTCCAGTCCATGTACTTCTTGATCTTGCATAGTACATTCAACCCTT 120
MB dugong   ............................C.........................T.... 120
CS1  H#10   ............................C.......................... .... 120
CS1  H#11   ............................C.......................... .... 120
CS1  H#13   .......................................................... .... 120
CS2  H#3    ............................C.......................... .... 120
Fl manatee ..CA..........C.T........TC.T.C......A.........C....CATG.T.C. 117

TS dugong   TA-TCGTACATAGCACATCTCT-GAGATAGTTCTCGTCAACACGCTTATCACCTCCAATG 178
MB dugong   ..............................C..T.......................... 178
CS1  H#10   ..............................C..T.......................... 178
CS1  H#11   .G.......................................................... 178
CS1  H#13   ...................................T.......................... 178
CS2  H#3    .G.......................................................... 178
Fl manatee ..A..............TA..T..A..CA.C..........T..............T.A 177

TS dugong   AACAGTCCTTGACTAC  194
MB dugong   ................  194
CS1  H#10   G...............  194
CS1  H#11   ................  194
CS1  H#13   G...............  194
CS2  H#3    ................  194
Fl manatee GG..........TC..  193
```

Figure 10.2: Alignment of a Torres Strait (TS) dugong (H#2) and a Moreton Bay (MB) dugong (H#18), to the three haplotypes of *Case Study 1* (CS1): H#10, #11 and H#13, the sample from *Case Study* 2 (CS2), H#3, and to the sequence of a Florida manatee (H#1). Refer to table 7.1 for an entire listing of the haplotypes observed as part of this genetic population structure study. The region of DNA sequence is the hypervariable region 1 (HVR-1) of the control region (or D-loop) of mitochondrial DNA (figure 6.1). The TS dugong is the reference sequence. Hyphens indicate a gap in the sequence, a period indicates common sequence. Differences from the reference sequence are indicated by the variable nucleotide. The numbers at the end of the sequence indicate position in HVR-1, omitting gaps in the sequence. The manatee is the closest related extant species to the dugong. It is nearly impossible to align DNA sequence from any other species in this region of DNA. There is therefore, no question that the samples: H#3, H#10, H#11 and H#13, are dugongs.

Section 19(1a) *Penalties and Sentences Act 1992*. Roberts *et al.* (1996) stated that although the required legislation is well developed in Australia, charges regarding the exploitation of natural resources such as in the above mentioned case, are usually dropped or no penalties enforced.

Incidents of non-prosecution reflect the enormous practical difficulties in policing large areas of remote country, and the fact that it is often inappropriate to enforce the law under these circumstances (Roberts *et al.*, 1996). Furthermore, cases are complicated by Section 93 of the *Queensland Nature Conservation Act 1992*, that provides for traditional (subsistence) use of natural resources by Aboriginal peoples and Torres Strait Islanders.

10.3 CASE STUDY 2: FROM WHICH REGION WAS THE DUGONG HUNTED?

The defendant was charged with the alleged breach of Section 88 of the *Queensland Nature Conservation Act 1992*. Information was received that the defendant was hunting for dugong without licence, permit or other authority, and was also selling the meat. A bag of meat and hunting apparatus were seized from the defendant's residence in November 1996 during a search warrant executed under the *Queensland Nature Conservation Act 1992*.

Upon visual inspection by Dr J. Miller (Department of Environment and Heritage, Queensland) the piece of meat was identified as dugong, and a frozen sample was delivered to James Cook University, Townsville, Queensland, in April 1997 for confirmation of the species of origin using DNA analysis. DNA was extracted from the tissue (section 4.1), amplified using the HVR-1 primers (section 4.3, table 4.1) and the PCR product directly sequenced (using automated sequencing) following methodology in section 4.4.

DNA sequencing of the HVR-1 confirmed that the piece of meat was dugong by comparative alignment with other dugong sequences (figure 10.2). The haplotype (H#3) has also been observed from sampled dugong populations of the Torres Strait and Broome (figure 10.1). The haplotype has not been observed in any dugong populations sampled southeast of the Torres Strait. The findings of this study support the defendant's statement

that the dugong was not hunted in the Townsville region, but from a location further north (i.e., Torres Strait). However, I cannot exclude the possibility that it was a dugong from Townsville. The progression of this case awaits the verdict of another similar case which will test whether native hunting rights are extinguished by legislation.

10.4 CONCLUSIONS

Sequence of the HVR-1 of mtDNA is a useful genetic signature for the dugong in situations of wildlife law enforcement. This genetic region is able to: 1) verify if the biological material is dugong, 2) indicate the minimum number of individuals, and 3) suggest the geographic origin.

CHAPTER ELEVEN

PHYLOGEOGRAPHY

In this chapter, divergence times (Chapter 8) and intraspecific dugong genetic structure (Chapter 7) are integrated with information on present-day distribution and palaeogeography, to produce a model explaining the genetic pattern exhibited by the mtDNA haplotypes. By recognising the geographical events and features that are likely to have contributed to the differentiation of the intraspecific dugong units, insight into factors affecting dugong distribution and dispersal are provided.

11.1 PALAEOGEOGRAPHY, PHYLOGENETICS & PHYLOGEOGRAPHY

Palaeogeography is the term used to describe the historical geography of an area on a geological time scale (figure 2.3). *Phylogenetics* is the term used to describe the evolutionary history and line of descent of a species or higher taxonomic group (Lawrence, 1989). *Phylogeography* is the term coined by Avise *et al.* (1987) to describe the association of genealogical lineages with their present day and historical geographic distribution. Differences in phylogeographic patterns between species are often associated with their vagility and with the past and present fragmentation of their environment (Avise, 1994).

It is probable that the most plesiomorphic (ancestral) dugong haplotypes have been lost (figure 11.1), or are unsampled by this study. Therefore, the actual divergence times may be older than the estimates presented in table 8.4. The evidence presented in Chapter 8 suggests the sampled dugong haplotypes diverged from a common ancestor around two to three million years (Myr) ago (table 8.4). Therefore, I predict that any barriers which physically separated dugong groups, would have been caused by climatic and geological events from the late Pliocene to Recent times (see figure 2.3 for geological time scale).

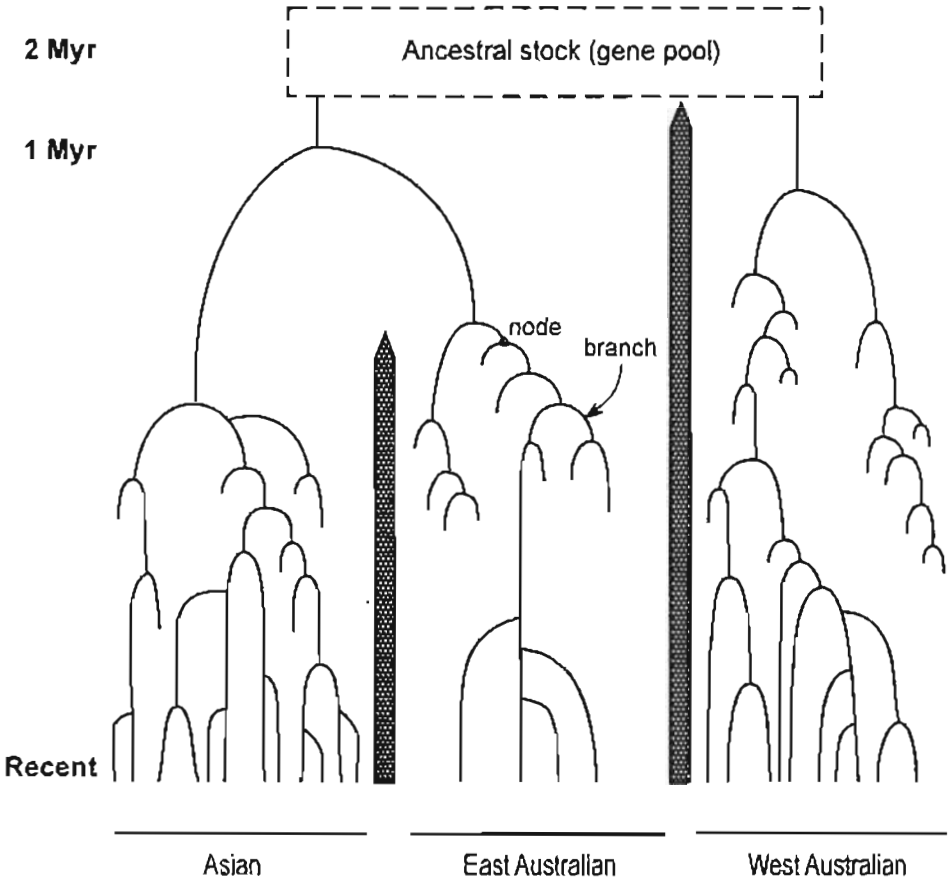

Figure 11.1: Speculative divergence dates and time scale to express the historical origins of each of the three mtDNA haplotype clusters. Extant haplotypes are presented at the terminals. Each node represents an individual matriline, with branches leading to daughter matrilines. The geographic distribution of the West Australian and East Australian haplotypes have overlapped after an extensive period of physical isolation. This figure is modified from Avise (1994).

Potential barriers which are likely to have contributed to historical fragmentation of the dugong's habitat around Australia include:

- Low sea level phases which reduced the continental shelf environment as a marine habitat and created a land bridge between Papua New Guinea and Australia, in the area of the Torres Strait.
- Prevailing current systems which are affected by changes in sea level.
- Variations of sea temperature with changes in sea level, which would have in turn affected the dugong's range (dugongs require regular access to water >16°C (Preen, 1993)).
- The *Java Trench-Timor Trough-Aru Basin* deep sea trenches at the edge of the northern continental margin.
- Associated ecological barriers. For example, changes in the distribution of seagrass on which dugongs are dependent.

11.2 PALAEOGEOGRAPHY AROUND THE NORTH AUSTRALIAN COAST

11.2.1 Geology and Geography

Geological barriers are created by tectonic events and sea level fluctuations, both of which are associated with climate change (Poore, 1994). Australia moved northwards during the Tertiary reaching its present orientation by the Quaternary (Davies, 1988; Poore, 1994). In the early Tertiary, the northern continental margin of Australia was in the vicinity of the central ranges of West Irian, and although the continental margin was alternately submerged and emergent, it was stable tectonically (Doutch, 1974). In the Miocene, deformation (collision of crustal plates) intensified and by the end of the Pliocene most of the present land mass of New Guinea had been created (Doutch, 1974). By the beginning of the Quaternary, the arrangement of land and sea in the south-west Pacific was essentially as it is today (with Torres Strait exposed as land) (Poore, 1994). Geological features formed by the collision of the continental plates between Australia and south-east Asia include the deep-sea trench of Java (subduction zone), and the Indonesian archipelago (separating the Indian Ocean from Malayan provinces) (figure 11.2). The Indonesian archipelago would have had greater effect as a barrier to dugong movements during periods of low sea level.

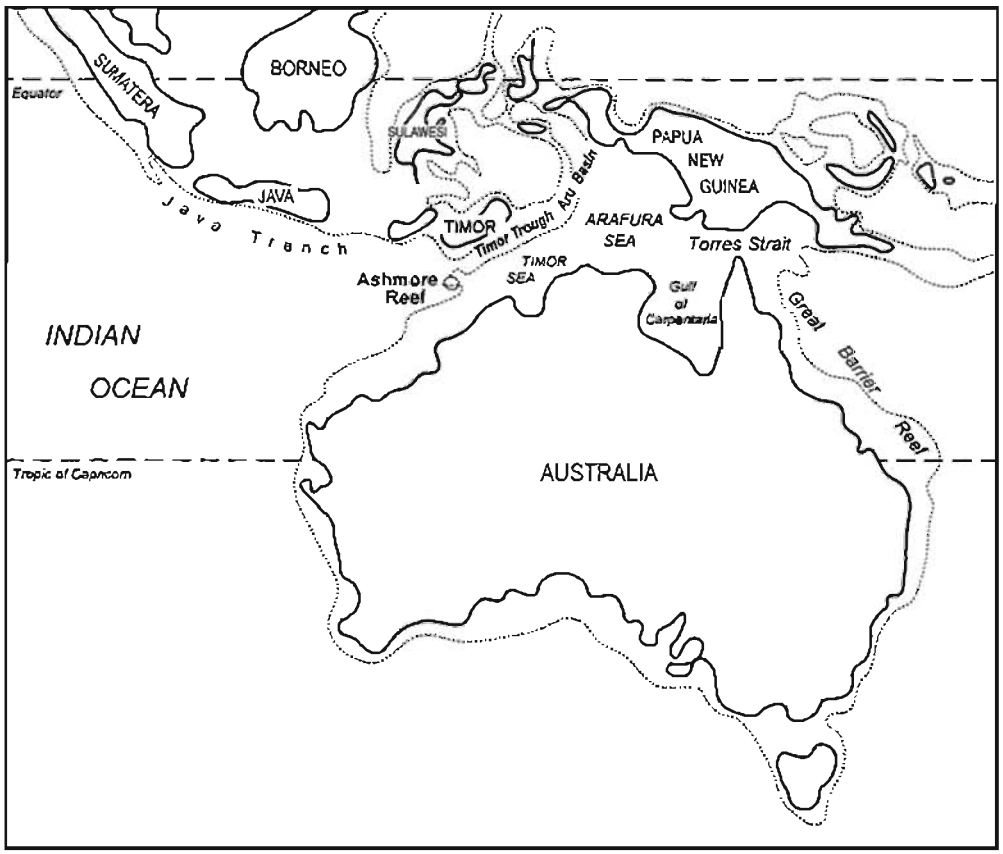

Figure 11.2: Geological features of Australia and the Indo-Pacific. The continental shelf is indicated by the 200 m bathymetric line. The Indonesian archipelago includes: Sumatera, Java and Timor, bordering a tectonic boundary indicated by the deep sea trenches: *Java Trench - Timor Trough - Aru Basin*.

The north Australian continental margin has been relatively stable tectonically since the Miocene. Therefore, the only major geographic changes to the marine shelf, were the Pleistocene sea level fluctuations accentuated by global palaeoclimatic cycles (i.e., Milankovitch-cycles (Veron, 1995)). During the Pleistocene, the sea level fluctuated more than 200 m with brief episodes of sea levels up to about 40 m higher than today (Galloway & Kemp, 1981; figure 11.3), and continually changed the extent and shape of the Australian coastline (Galloway & Kemp, 1981). The numerous periods of low sea levels substantially reduced the size of marine habitats on the continental shelf (figure 11.2) (Poore, 1994). Although the Arafura Sea (figure 11.2) was probably inundated in the late Tertiary, it would have been land during times of low sea level associated with the Pleistocene glaciations (Doutch, 1974). The Gulf of Carpentaria (figure 11.2) probably formed by local downfaulting late in the Pleistocene (Doutch, 1974). Torres Strait (figure 11.2) is unlikely to have been a marine passage until the middle to late Pleistocene and then would have opened and closed repeatedly with the sea level fluctuations (Doutch, 1974; Chappell & Shackleton, 1986; Poore, 1994).

The existence of Torres Strait as a seaway has been more the exception than the rule, with each glacial phase existing for about 100,000 years, and each interglacial phase for a duration of only about 10,000 years (figure 11.3; Poore, 1994). Torres Strait most recently became a seaway around 6,000 years ago (figure 11.4; Galloway & Kemp, 1981; Pirazzoli & Pluet, 1991). The alternation between glacial and interglacial environments during the Pleistocene was much more rapid than any changes experienced in Australia during the Mesozoic and earlier Cenozoic times (Williams, 1984).

11.2.2 Biological implications

Any barriers associated with historical changes in sea level are likely causes of population subdivision of inshore fauna. By controlling the sea level, glaciations change coastal geography, influence current systems and water temperatures (Chappell & Shackleton, 1986; Poore, 1994). In combination, currents and temperature can function as a physiological barrier, maintaining discrete populations of species which are somewhat confined to separate current systems (Schopf, 1979).

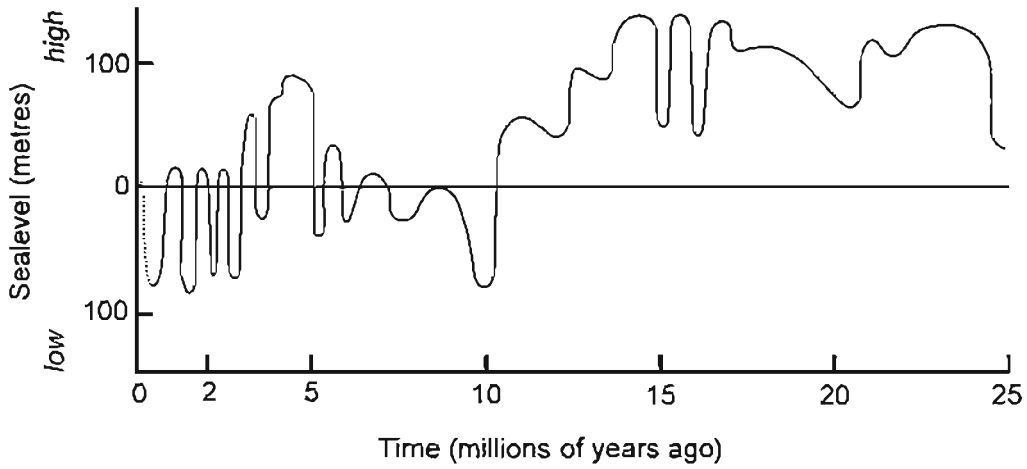

Figure 11.3: Sea level curve. A schematic representation of the sea-level curve of Vail & Hardenbol (1979), which has been adapted to indicate that the curve meets +0 m sea level for the present day (Zann, 1996). Sea level rise (transgression) and fall (regression) in a coastal area results from the melting or formation of the polar ice caps causing a change in the ocean's volume (eustatic changes) (Zann, 1996).

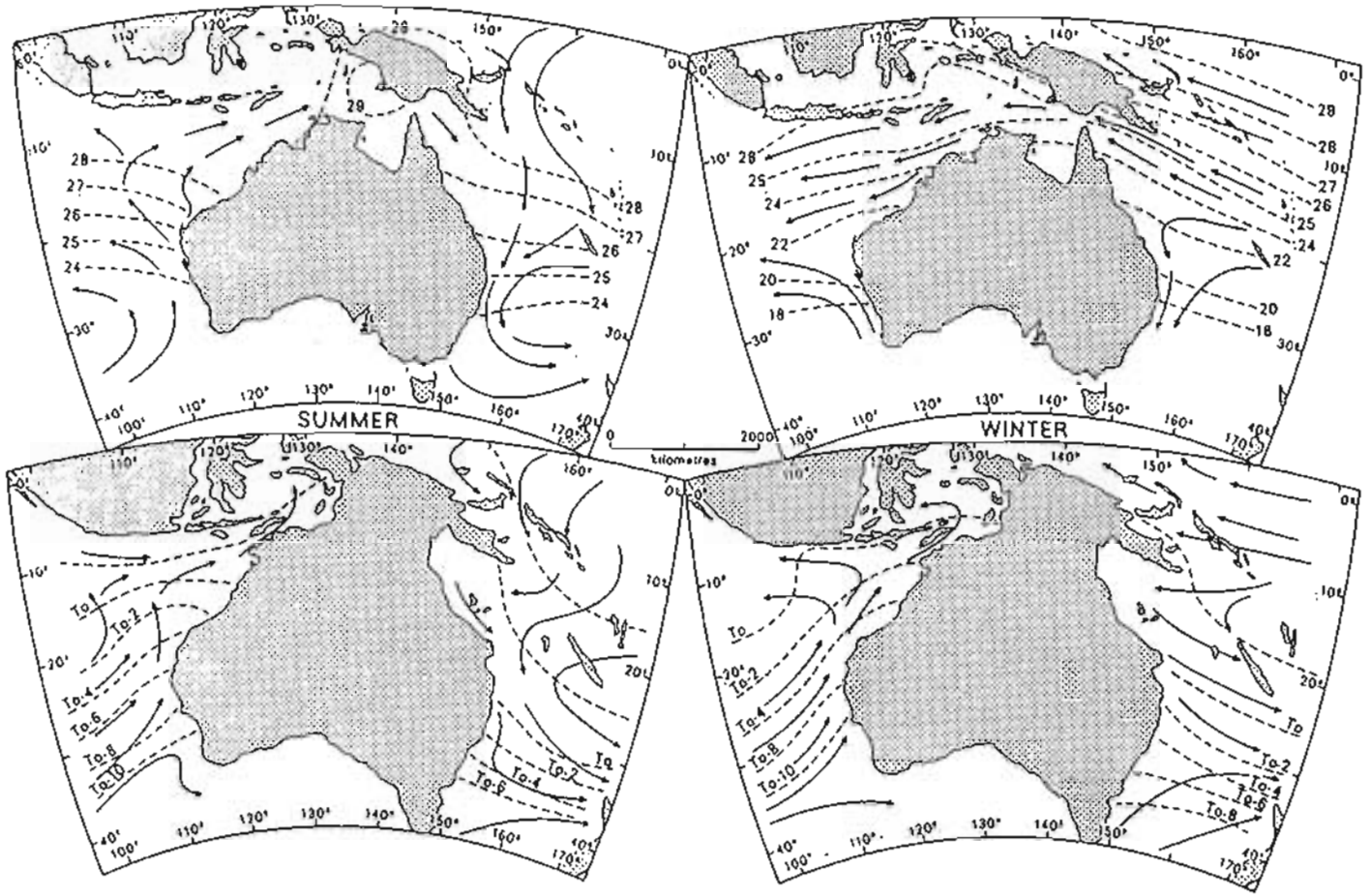

Figure 11.4: Summer and winter surface currents of the Australian region. The upper charts refer to the present distributions, whereas the lower charts indicate a tentative reconstruction of the distributions during the maximum of the last glaciation. Figure taken from Webster & Streten (1974).

During periods of high sea level when the submerged shelf area was greatest, temperatures were higher (similar to the modern climate). Conversely, during glacial periods and low sea level, sea temperatures were lower. Long term exposure to moderately cold water temperatures (less than 16°C) have been recorded as a cause of death in Florida manatees (O'Shea *et al*, 1985; Ackerman *et al.*, 1995), and although dugongs are not known to perish because of low water temperatures, they will actively avoid temperatures lower than 19°C and need access to temperatures greater than 16°C (Preen, 1993). The limited shelf habitat availability and the prevailing climatic and environmental conditions of glacial periods, would have severely restricted the distribution of tropical and subtropical species (Poore, 1994), including the dugong and its seagrass food.

Pockets of suitable dugong habitat around the Australian continental shelf are likely to have existed even at periods of low sea level and low temperatures. For example, according to the most recent estimate by Davies (1997), the Great Barrier Reef (on the east coast of Australia) was initiated 400,000 years ago. Core samples indicate the Great Barrier Reef is composed of five superimposed layers, which grew in periods of sea level highstand. Each reef was killed by a succeeding glacially-induced fall in sea level (Davies, 1997). Apparently the most recent reef has been in existence for only the last 10,000 years (the Holocene (Davies, 1988)). Obviously the reef has survived episodes of destruction and regeneration during alternating phases of low and high sea levels respectively. As small embayments for shelf-inhabiting species would have existed even during periods of low sea level, it seems likely that these surviving pockets ('nurseries') of coral reef on the Australian continental margin, would have been the most likely source for the regeneration of the Great Barrier Reef (Davies, 1988). Similar refugia for seagrass are also likely. Specht (1981) suggested that seagrass co-distributed with mangrove fauna, and colonised the Australian coastline in the early Cretaceous. Geological evidence indicating the specific location and extent of seagrass refugia on the Australian continental margin during low sea level phases, is lacking (Poore, 1994). However, there is evidence in fossil assemblages at Shark Bay, Western Australia, suggesting the presence of seagrasses since the Pleistocene (Logan *et al.*, 1970).

Seagrass colonisation would have preceded dugong colonisation, therefore suitable dugong habitat may have existed on the Australian subtropical to tropical coastline since the early Cretaceous. The only convincing records of

dugong fossils are from Papua New Guinea, and are dated as Pleistocene in age (section 2.4.1). The discovery of dugong subfossils south of their present Australian range on the coast of New South Wales (Etheridge *et al.*, 1897; Etheridge, 1905), dates their existence on the Australian coastline to at least 100,000 years before present (i.e., the last high sea level phase). However, these subfossils do not necessarily indicate the historical dugong range. The presence of subfossils beyond the recognised dugong range may reflect the movements of dugongs in response to a major habitat disturbance similar to that in Hervey Bay in 1993 (Preen & Marsh, 1995). Nevertheless, there is an indication of dugong colonisation on the Australian coastline at least since 100,000 years ago. It is possible dugongs were on the Australian coastline before the Pleistocene, but there is no fossil evidence to support this.

11.3 DUGONG PHYLOGEOGRAPHY

The genetic marker employed in this study is maternally inherited, therefore the interpretation regarding dugong phylogeography is female mediated. The interpretations I have made from the genetic data may be confounded by the small sample sizes for the West Indian Ocean (n=4) and Asian (n=7) geographic units. However, as the genetic diversity measures include both haplotype and nucleotide diversity, I have assumed the estimated genetic diversities are indicative of the actual genetic diversity and relationship among geographic units (see section 3.9 for discussion on this).

Phylogenetic trees constructed from the mtDNA haplotypes in Chapter 7 consistently recognised three haplotype clusters for the dugong: West Australian, East Australian, and Asian (figure 7.4). Only the Asian samples constituted a natural phylogeographic unit (see Chapter 7), being distinct both genetically and geographically from all others. Where genetically distinct clusters are not geographically isolated, I have investigated possible causes and have predicted divergence dates.

Allele (e.g., haplotype) coalescence (Hudson, 1990) and phylogeography (Avise *et al.*, 1987) use information on haplotype relationships as well as their distribution to make inferences about population structure and history (Hillis *et al.*, 1996). Haplotype coalescence assumes that the more similar the haplotypes are in DNA sequence, the less divergent they are (i.e., more

closely related). Using coalescence models (Hillis *et al.*, 1996), I estimated the expected times since common ancestry among dugong haplotype groups (table 8.4).

In the context of the palaeogeography of the Indo-Pacific, and bearing in mind the strong support for the three main haplotype clusters, with weak support for the arrangement of the haplotypes within each of the three clusters, I will now explore possible scenarios to explain the emergence of the genetic pattern shown by the mtDNA haplotypes, addressing the following observations:

1) Despite their considerable geographical separation, the haplotypes of West Indian Ocean dugongs are extremely similar to haplotypes from the East Australian dugong populations. One haplotype (H) is shared between these regions (H#15, figure 7.4).
2) The Asian and East Australian haplotype clusters are more closely related to each other (share a more recent ancestor), than either is to the West Australian haplotype cluster.
3) Asian dugongs share no haplotypes with Australian or West Indian Ocean dugong populations.
4) West Australian and East Australian haplotype clusters overlap in the region of the Great Barrier Reef.

I deal with each of these observations separately below.

1) Despite their extensive geographical separation, the haplotypes of West Indian Ocean dugongs are extremely similar to haplotypes from the East Australian dugong populations. One haplotype (H) is shared between these regions (H#15, figure 7.4).

Of the four West Indian Ocean samples, one individual was found to have haplotype #15, whereas the remaining three samples had the common closely related haplotype #35 (table 7.1). Haplotype #15 also occurred in East Australian dugong populations with high frequency. The most common (and widespread) haplotype in a present day population is probably a plesiomorphic (ancestral) haplotype that has managed to squeeze through one or more bottlenecks (Avise *et al.*, 1979). Haplotype #15 (table 7.1) is the most commonly occurring and widespread haplotype in the dugong sample (figure 7.2).

The similarity between the haplotypes of West Indian Ocean dugong populations and those of East Australia does not necessarily indicate that these dugongs interbreed. More likely, dugong populations of the West Indian Ocean and East Australia were founded from a common source population in recent evolutionary time.

2) The Asian and East Australian haplotype clusters are more closely related to each other (share a more recent ancestor), than either is to the West Australian haplotype cluster.

On the basis of haplotype coalescence, the estimated divergence from a common population between West Australian and Asian haplotype clusters was about 2.5 Myr ago (table 8.4). This time of divergence coincides with the end of the Pliocene when the Indonesian archipelago, the (*Java Trench - Timor Trough - Aru Basin*) deep sea trench, and the Torres Strait as a land bridge, were already in existence. Any of these geological features alone or in combination with other barriers (accompanying changes in current systems, temperature, ecology) may have acted to separate the dugong populations of North & West Australia and Asia.

The present current system is expected to be mirrored during other periods of high sea level (Pleistocene glacial phases) (figure 11.5, Webster & Streton, 1974). Current systems are less imposing than geological features (McManus, 1985), and are not considered as 'physical' barriers to dugong movements. I consider it unlikely that currents are preventing movement of dugongs between the west coast of Australia and south east Asia. However, currents may have played a role in dictating the direction of dugong migrations. Pleistocene current systems at high sea level (i.e., similar to the present) support movements for a founding population from Asia to the east coast of Australia via the north coast of Papua New Guinea (figure 11.4). Haplotypes intermediate between those of Asia and East Australia may exist along the northern coast of Papua New Guinea. Unfortunately, samples to test this hypothesis were not available to this study.

3) Asian dugongs share no haplotypes with Australian or West Indian Ocean dugong populations.

Often very different faunas occur across a tectonic boundary. Such 'plate effects' accumulate over time (Palumbi, 1994; Valentine, 1973). Tectonic boundaries separate both West Indian Ocean and Australian dugongs from

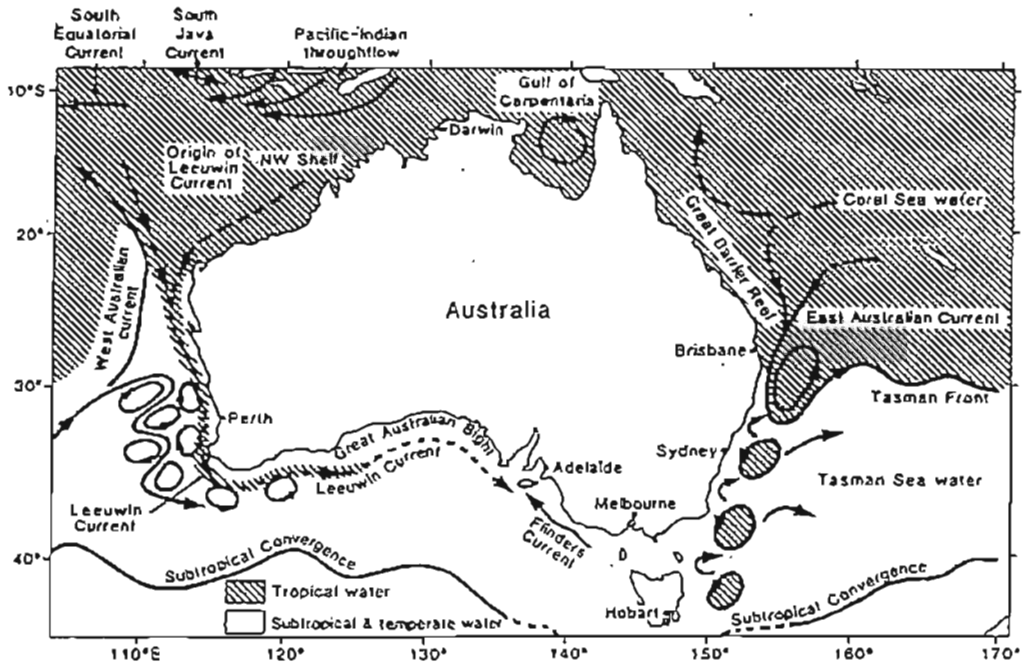

Figure 11.5: Major surface currents of the Australian region. Taken from Jeffrey & Hallegraeff (1990).

Asian dugongs (figure 11.2). Strong genetic partitioning across the Indonesian archipelago has been observed in a number of species as well as in the dugong: bryozoans (Schopf, 1976), coconut crab (Lavery *et al.*, 1995; 1996) and the Hawksbill turtle (Broderick *et al.*, 1994).

Australian and West Indian Ocean dugong populations have an essentially continuous, linear and (perhaps more stable) coastal distribution, in comparison to the patchy island distribution of Asian dugongs. The evolutionary consequence of population crashes, local extinctions and colonisations for a patchily distributed species in a dynamic environment, may over the long term increase the variance among populations. Laboratory breeding experiments with *Drosophila* have shown that environmental heterogeneity is positively correlated with genetic diversity (Powell, 1971; McDonald & Ayala, 1974). The biodiversity of the marine environment of southeast Asia is the highest in the world (McManus, 1985). The greatest range of habitat variability occurs along irregular shorelines of high islands which are common throughout southeast Asia, especially in the Philippines and eastern Indonesia (McManus, 1985). The marine diversity of Asia can be accounted for by the successive periods of isolation and species remixing, controlled by intermittent land connections, varying with sea level and tectonic changes (McManus, 1985).

If we consider the dispersal ability of the dugong (section 2.4.2), present day geographical features are not convincing as barriers to dugong movements. Perhaps deep sea trenches, current systems, and the limited continental shelf of Papua New Guinea, should be credited with playing a greater role in restricting Recent dugong dispersal and interactions among populations. Alternatively, the geographical features that played a role in separating dugong groups at low sea level, may no longer be physical barriers. Dugong behaviour and regional differences in reproductive readiness and life history may have evolved since their separation (microevolution), and become so significant that the previously separated dugong populations (i.e., from Asia and Australia), remain allopatric despite their geographic proximity.

It is reasonable to consider whether dugong behaviour plays a major role in determining patterns of dispersal and interaction among populations. Dugong movements are highly individualistic (figure 2.2; section 2.4.2) and the factors determining their movements is unknown, other than their

avoidance of cold water (section 2.4.2), emigration from an area when food availability is diminished, or moving to an area where other dugongs are feeding (section 2.4.2). The reason why these geographically proximal dugongs (i.e., Asian and Australian) are not interbreeding may be simply due to behavioural barriers. That is, dugongs may be relatively philopatric.

An interesting result, not included in the analysis as it arrived late in the progress of this study, is a sequence obtained for an Ashmore Reef (12°15'S, 123°05'E) dugong (Appendix G). Even though the Ashmore Reef haplotype is unique, it is of the Asian type rather than Australian. Ashmore Reef is on the edge of the Sahul shelf (Australian), which is also on the Australian continental shelf side of a deep sea trench dividing the Australian continental shelf from Timor, and is a mere 250 km from the north Australian coastline (figure 11.2). The fact that the Ashmore Reef dugong is Asian-typed rather than Australian suggests that deep water is not an impermeable barrier to dugong movement. It may be that a different dispersal behaviours exist for Asian and Australian dugongs. Perhaps, because of their archipelagic distribution, dugongs of the Asian type are more accustomed to crossing deep water and are less influenced by currents, than the coastal-hugging dugongs of Australia.

4) West Australian and East Australian haplotype clusters overlap in the region of the Great Barrier Reef.

It is interesting that the geographic distributions of the two Australian haplotype clusters coincide with the tropical biogeographic provinces discussed by Knox (1963) and Wilson & Gillett (1974). The Dampierian Province ranges from north-western Australia (about 25°S to 28°S) to the Torres Strait, and the Solanderian Province covers the coast of Queensland north of about 25°S to the Torres Strait (figure 11.6). Although definition of these biogeographic provinces is based on a single taxonomic group (molluscs), they have never been seriously disputed (Knox, 1963; Wilson & Gillett, 1974; Poore, 1994; 1995).

The geographic overlap of the two Australian haplotype clusters, East Australian and West Australian (figure 7.4), would have occurred during the Pleistocene to Recent, and only during the short periods (geologically) when the Torres Strait was a seaway (during periods of higher sea level, figure 11.4). The most recent opening of the Torres Strait as a seaway, was 6,000 years ago.

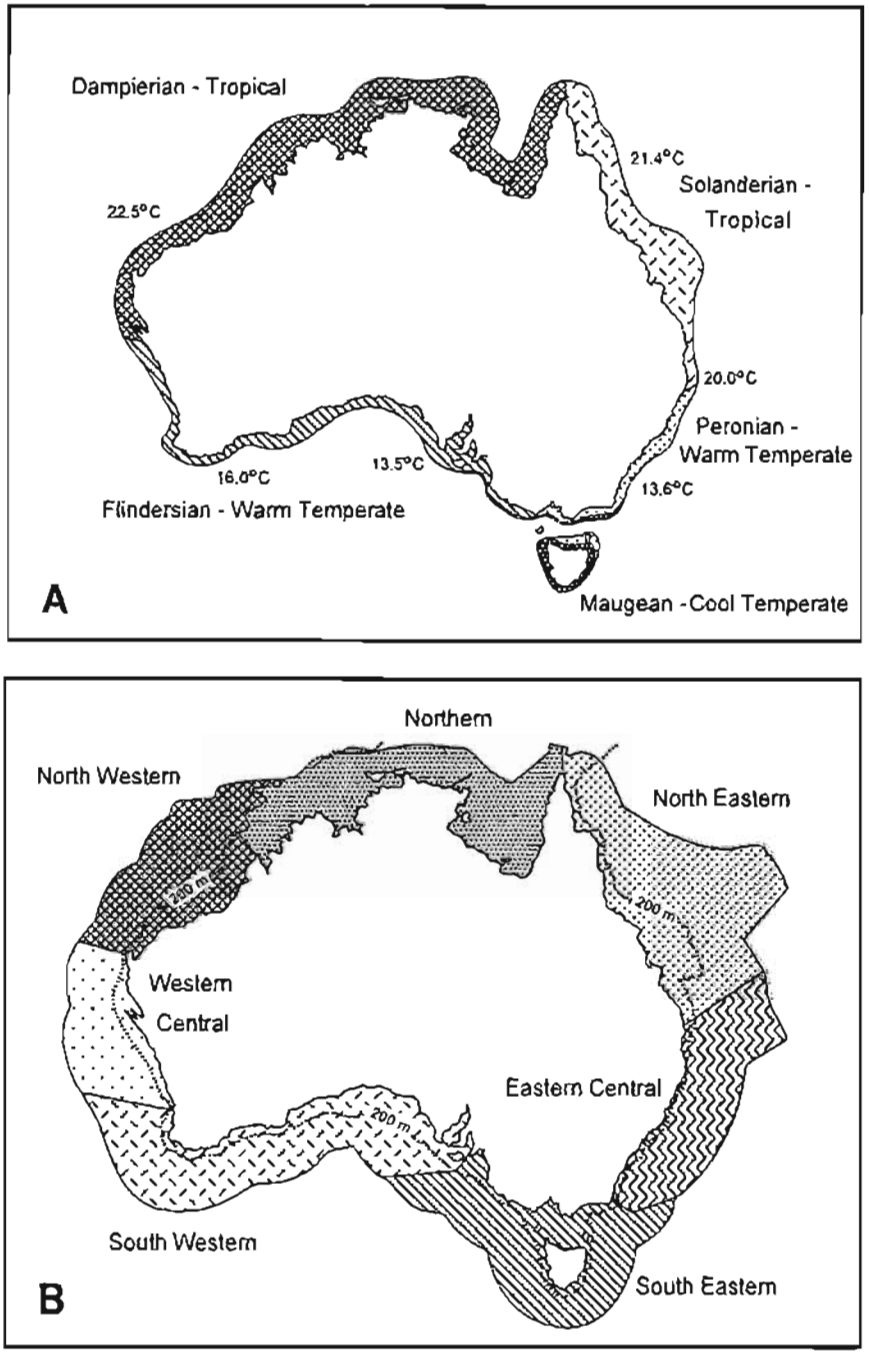

Figure 11.6: Biogeographic provinces and Large Marine Management Regions of Australia. A) Biogeographic provinces of the Australian intertidal area, according to Bennett & Pope (taken from Poore, 1995). B) Coastal zones within the 200 m bathymetric contour and the 200 nautical mile Exclusive Economic Zone adopted by the Australian Committee of the IUCN in 1986 (Poore, 1995), which is also referred to as the 'Large Marine Management Regions of Australia' by the Commonwealth Science and Industry Research Organisation.

It is of particular interest that the spread and overlap of these two haplotype groups around the Australian coastline have not been more extensive. Typical West Australian haplotypes occur in dugong populations almost as far as the southeast Australian extreme of the dugong's range. For example, 0.08% of the haplotypes in the Hervey Bay population are West Australian (figure 7.5). However, typical East Australian haplotypes do not extend north and west along the Australian coast beyond the Torres Strait (figure 7.5). In addition, the presence of subdivision within a haplotype cluster (section 7.2.3) suggests female philopatry.

11.4 CONCLUSIONS

The lack of geographic overlap of Asian and Australian haplotypes indicates that these dugong populations are reproductively isolated. Reproductive isolation can be caused by any of the following potential barriers to dugong movement: 1) geography (e.g., the Torres Strait land bridge), 2) hydrography (temperature and current systems), and/or 3) behaviour.

The two Australian haplotype clusters overlap in the region of the Great Barrier Reef. The divergence of the two Australian genetic units may be attributed to the periodic land bridge between Australia and Papua New Guinea during the Pleistocene glaciations. Considering the Torres Strait has been a seaway for the last 6000 years, and the dispersal ability of the dugong, it is of interest to dugong conservation management that the overlap of the two Australian haplotype clusters is not more extensive.

CHAPTER TWELVE

GENERAL DISCUSSION

12.1 OVERVIEW

This chapter discusses the use of genetic data as an aid to the conservation management of the dugong at both global and regional scales. I address the reasons why we should not be complacent about the dugong's conservation status, despite its estimated global population of more than 100,000, extensive range, relatively high intraspecific genetic diversity, and occurrence as only one Evolutionarily Significant Unit (ESU).

I recommend that units for dugong management be based on a chain of sanctuaries comprising prime dugong habitat. The presence of genetic substructure among Australian populations at a scale of several thousand kilometres, suggests that management should be developed at a regional scale. As the rate of gene flow among neighbouring populations is presumed to be low, I also recommend introducing measures aimed at maintaining the connectedness of the dugong populations as a precautionary management principle. The degree of connectivity between neighbouring populations needs further investigation, especially by studying the movements of individual dugongs. In order to obtain a complete picture of the genetic structure of the dugong, future studies should include using a nuclear marker to examine the genetic structure of male dugongs, and possible sex specific differences in dispersal.

12.2 THE DUGONG AS A SPECIES WHICH DESERVES SPECIAL CONSERVATION STATUS

The dugong meets four of the five criteria suggested by Noss (1990) as warranting special conservation status for a marine species:

1) **Keystone species**: a pivotal species upon which the diversity of a large component of the community depends. Dugongs are considered pivotal to the seagrass communities (including seagrass

dependent fauna) on which they feed (Preen, 1995b; Poiner & Peterken, 1995).

2) **Umbrella species**: species with large area requirements which given sufficient protected habitat area, will bring many other species under protection. The range of individual dugongs is large (Marsh & Rathbun, 1990; A.R. Preen, personal communication). Prime dugong habitats comprise inshore seagrass areas inhabited by several other species of marine wildlife including cetaceans and marine turtles, as well as commercially important crustaceans (prawns/shrimp) (Poiner & Peterken, 1995).
3) **Flagship species**: popular species that serve as rallying points for major conservation initiatives. Dugongs have received icon status recently as 'charismatic megafauna'. For example, the site of a development at Oyster Point, Cardwell (Australia), fringing the Great Barrier Reef World Heritage area, is frequented by the dugong. The possible anthropogenic impacts of the development are of great concern to conservationists, and the potential impact on dugongs has been a focus of their campaign.
4) **Vulnerable species**: those species which are prone to extinction. The dugong is listed by the IUCN (1996) as 'vulnerable to extinction'.

12.3 DIFFICULTY IN DETERMINING CONSERVATION STATUS OF MARINE SPECIES

Many of the management issues for organisms in the terrestrial environment are inappropriate for marine species, because of the connectivity (openness), large geographic scale, and unfamiliarity of marine systems (Kenchington, 1990). Thus many of the indications that a species is secure, such as large range, large population size, and high genetic diversity may not be reliable in the case of marine species as discussed below.

12.3.1 Large Geographic Range & Viable Population Size

At least 15 marine and coastal animal species have become extinct in Recent times (Vermeij, 1993). Five of the 15 extinct marine and coastal species had large geographic ranges (Vermeij, 1993), challenging the widely accepted generalisation among conservation biologists and palaeontologists, that species with large ranges are not prone to extinction. One of these species

was the dugong's closest relative, the Steller's sea cow. In addition to similarities in biology and life history (e.g., large, slow breeding marine mammals), both species had their numbers reduced by direct harvest, require high adult survivorship for population maintenance, are (were) specialised feeders, and had extensive ranges in inshore coastal regions.

A relatively large population size is also not indicative of secure status. The minimum viable population size for terrestrial animals is usually based on the '50-500 rule' (the 'Franklin-Soulé number', Franklin, 1980; Soulé, 1980). The Franklin-Soulé number is a minimum intended to maintain sufficient numbers of breeding adults to avoid the erosion of the genetic 'robustness' of the gene pool of a species. Inbreeding and loss of genetic variation can, in theory, lead to extinction in closed populations. Ralls *et al.* (1979) demonstrated that inbreeding often leads to increased juvenile mortality in small ungulate populations. For short-term fitness, the critical minimum number of breeding adults (or effective population size, N_e) required to avoid loss of genetic variation from inbreeding effects, is allegedly 50. Five hundred breeding adults are theoretically needed to maintain the level of genetic variation required for adaptation to a change in environmental conditions, that is, to avoid the loss of genetic variation due to genetic drift (Franklin, 1980). For long-term evolutionary potential, population size should be much larger (Shaffer, 1981). Lande (1995) recommended an N_e of at least 5000 to ensure the long-term viability of a species. However, the 'actual' population size (N) may be five times larger than N_e depending on the number of actively breeding individuals.

The 50-500 rule is not universally accepted for terrestrial species, and is not considered a useful concept for the open populations of marine organisms (Jones & Kaly, 1995). More important than population size, is the recognition of decline and the rate at which it is occurring. For example, despite the discontinuous and extensive range of the African elephant (*Loxodonta africana*) of 7 million km^2 south of the Sahara, the 1979 population estimate of 1.3 million was halved by 1987 (review by Caughley & Gunn, 1996). The large range of this species was not a reliable index of secure status, whereas a rapid rate of decline provoked serious conservation concern.

The global population size for the dugong is predicted to be greater than 100,000 (see tables 2.3 & 2.4 for Australian and international population

estimates respectively). Anecdotal reports indicate a widespread decline in dugong numbers and some evidence of local extinction. Along some 2000 km of the southern Great Barrier Reef (GBR) coastline, aerial surveys confirm a 50% reduction of dugong population size between 1986/87 and 1994 (Marsh *et al.*, 1996). The genetic structuring of dugong populations along the east Australian coast, and the suggestion of a low level of female migration (Nm) between neighbouring populations, suggests that the dugong decline in the southern GBR is unlikely to be due to emigration to other, more northern dugong populations such as the Torres Strait as claimed by fishers (T. Loveday personal communication to H.D. Marsh).

12.3.2 Genetic diversity

Maintaining intraspecific genetic variation is an important issue in conservation management, as genetic variation is considered an index of evolutionary potential (Soulé, 1980; Allendorf & Leary, 1986). However, as reviewed in Chapter 2, low genetic diversity is the norm for some species, and appears in many cases to be the outcome rather than the cause of population bottlenecks (Avise, 1994). The cost of inbreeding to genetic fitness, and the ability to recover from a population bottleneck, are species-specific characteristics and generalisations are not possible (Avise, 1994). That is, low genetic diversity is not necessarily synonymous with poor genetic health, nor is high genetic diversity a prerequisite for species survival. For example, the recovery of populations of the northern elephant seal (*Mirounga angustirostris*) does not appear to have been affected by its low genetic variation (Bonnell & Selander, 1974). On the other hand, the low genetic diversity at the major histocompatibility complex (MHC) gene of cheetah (*Acinonyx jubatus*) populations, has been linked with inbreeding and diminished fitness by lowering disease resistance (O'Brien *et al.*, 1985; O'Brien & Evermann, 1988).

Not all species that are rare or endangered have low genetic diversity. Despite fewer than 80 one-horned rhinoceros (*Rhinoceros unicornis*) in Nepal after 1962, they have one of the highest allozyme heterozygosities (near 10%) reported for vertebrates (Dinerstein & McCracken, 1990). The intraspecific genetic diversity of sirenians appears to be relatively high (table 12.1) which is unexpected for such large, slow breeding marine mammals (section 2.4.5; Martin & Palumbi, 1993).

Table 12.1: Comparison of mtDNA genetic diversity among various marine animals. Examples of the genetic diversity measured for the mtDNA of various marine animals. The nucleotide diversities are averages among haplotypes expressed as a percentage. Nucleotide and haplotype diversities were calculated using methods of Nei & Tajima (1981; 1983) and Nei (1987). In rapidly evolving genomes, haplotype diversity sometimes approaches 1.0 (Avise *et al.*, 1989; Avise, 1994). Hypervariable region 1 is abbreviated as HVR-1.

Species	Genetic marker	Sample size (n)	Genetic diversity		Reference
			% Nucleotide diversity (π)	Haplotype diversity (h)	
Dugong (*Dugong dugon*)	**mtDNA HVR-1 sequence**	**103**	**3.425**	**0.766**	**this study**
Florida manatee (*Trichechus manatus*)	mtDNA HVR-1 sequence	87	4.140	0.835	Garcia-Rodriguez *et al.* (submitted)
Amazonian manatee (*Trichechus inunguis*)	mtDNA HVR-1 sequence	14	0.500	0.875	Garcia-Rodriguez *et al.* (submitted)
Spinner dolphin (*Stenella longirostris*)	RFLP total mtDNA	90	1.175	-	Dizon *et al.* (1991)
Harbour porpoise (*Phocoena phocoena*)	RFLP total mtDNA	204	0.364	0.810	Wang *et al.* (1996)
Dall's porpoise (*Phocoenoides dalli*)	RFLP total mtDNA	101	0.550	0.930	McMillan & Bermingham (1996)
Narwhal (*Monodon monoceros*)	RFLP & sequences of mtDNA control region	74	0.170	0.006	Palsboll *et al.* (1997)
Humpback whale (*Megaptera novaeangliae*)	RFLP total mtDNA	84	0.402	0.105	Baker *et al.* (1990); Wang *et al.* (1996)
Humpback whale (*Megaptera novaeangliae*)	mtDNA control region sequence	90	3.000	-	Baker *et al.* (1993)
Killer whale (*Orcinus orca*)	mtDNA control region sequence	8	0.200	-	Hoelzel & Dover (1991)
Hawksbill turtle (*Eretmochelys imbricata*)	mtDNA control region sequence	103	1.190	0.849	Bass *et al.* (1996)
Green turtle (*Chelonia mydas*)	mtDNA control region sequence	147	0.500	0.830	Encalada *et al.* (1996)

In wild populations, genetic diversity is retained by maintaining effective population sizes at a level above that at which harmful levels of inbreeding may occur (i.e., the Franklin-Soulé number). This includes the protection of 'corridors' to permit gene flow among populations (Avise, 1994; section 12.7). Genetic diversity should be maintained automatically if intraspecific units are recognised and used as the basis for management. Recognising intraspecific subdivision and identifying phylogeographic structure should be a focus for management efforts.

Despite the apparent overall high genetic diversity of sirenians, the available data suggest that the peripheral populations of dugong are characterised by relatively lower genetic diversity as illustrated by the peripheral populations, Moreton and Hervey Bays in eastern Australia (table 7.5).

12.3.3 Reliable indicators of dugong status

If species range, population size and level of genetic diversity, are not necessarily good indicators of the status of a marine species such as the dugong, then what indicators should alert managers to a conservation requirement? The most effective criteria should enable assessment of the species at a national or regional, rather than global scales, as it is at the former scales that management is implemented. Gärdenfors (1996) recognised additional criteria that should be considered in order to assess species and identify conservation priorities from a national or regional perspective including ecology, life history, taxonomy, genetics, and aesthetic importance. However, even though Gärdenfors (1996) addresses the problems associated with applying the global status categories of the IUCN (1996) at a regional scale, and requests that the conservation manager consider a number of criteria (section 12.5), no assessment or action for fullfilling those criteria were presented.

The dugong is a large marine mammal with an extensive coastal range, a long generation time, low fecundity (Marsh, 1995), and a specialised diet (Johnstone & Hudson, 1981; Marsh *et al.*, 1982; Lanyon *et al.*, 1989). Considering these life history characteristics, the dugong was scored against the ranking system of Millsap *et al.* (1990) which indicates the degree of biological vulnerability and corresponding action, for species at a national scale (table 12.2). Considering these life history characteristics, the dugong

Table 12.2: Biological vulnerability and corresponding action. The relevant categories met by the data available for the dugong under Assignments, are taken from Table 1 of Millsap *et al.* (1990). The total score of 30.6 suggests the dugong be considered as a candidate taxon for management attention according to the assessment of these criteria by Millsap *et al.* (1990).

Biological variable	*Rationale*	*Assignment*
1. **Population size.** The number of adults throughout the range of the taxon.	The sum of the aerial survey population estimates of the various regions of Australia is approximately 85,000, of which about 50% are likely to be adult (Marsh, 1980). This figure is expected to be an underestimate.	(f) > 50,000 (0 points).
2. **Population trend.** Overall trend in number of individuals throughout the taxon's range over the last two decades (or other approximate time interval considering taxon's generation time).	Aerial survey data and anecdotal evidence suggests that the Australian dugong population size is decreasing.	(b) Population size believed to be decreasing (8 points).
3. **Range size.** The size of area over which the taxon is distributed during the season when distribution is most restricted.	The area surveyed for Australian dugongs totals 190,000 km^2. This is expected to be an overestimate of the area of occupancy within a survey area. The west coast of the Gulf of Carpentaria and the Kimberley coast are yet to be surveyed.	(d) 80,000 to 1 million km^2 (4 points).
4. **Distribution trend.** % change (since European settlement) in area occupied by the taxon.	There is some local scale evidence of change in the area of occupancy since 1788. However, there is no evidence of large changes in range.	(d) Area occupied has declined by 1 to 24% (2 points).
5. **Population concentration.** Degree to which individuals within populations congregate or aggregate seasonally.	Not applicable.	(d) Does not concentrate seasonally (0 points).
6A. **Average number of live young produced per adult female per year.** Ability of the taxon to recover from serious declines in population size.	The interbirth interval for dugongs is three to six years (Marsh *et al.*, 1984a; Marsh, 1995).	(a) < 1 offspring per female per year (5 points).
6B. **Minimum age at which females typically first reproduce.**	The pre-reproductive period of a female dugong is 10 to 17 years (Marsh *et al.*, 1984a; Marsh, 1995).	(b) > 8 years (5 points).
7A. **Dietary specialisation.** Degree to which the taxon is dependent on certain environmental factors.	Although the seagrass in Hervey Bay recovered somewhat after its destruction in 1992, counts of calves were extremely low when checked in 1994, indicating that reproduction was suppressed (Preen & Marsh, 1995).	(a) Number of individuals declines; no substantial shift in diet (3.3 points).
7B. **Reproductive specialisation.** Choices below relate to the primary way in which local populations respond to decreases in availability of preferred breeding sites.	Anecdotal information (Marsh *et al.*, 1984) suggests that dugongs may prefer to calve in shallow water to avoid shark predation. Inshore calving sites have probably been lost due to coastal development. The impact on dugongs is unknown. However, it is unlikely dugongs have shifted to other sites (as they are unavailable).	(a) Number of individuals declines (3.3 points).
7C. **Other reproductive specialisations.** Ecological or behavioural specialisations not covered in variables 7A or 7B.	Temperature requirements are known to be relevant to dugong movements (Preen, 1993).	(c) Not specialised (0 points).

Table 12.3: Potentially vulnerable marine species. The dugong is considered here, under each of the criteria for marine species that are particularly vulnerable as listed by Jones & Kaly (1996).

Criteria	*Relevant data on dugong*	*Conclusion*
Species with restricted geographic ranges. These species may suffer global extinction from relatively small-scale impacts.	The dugong has an extensive range. Infrequent regional extinction is unlikely to cause global extinction.	Not applicable
Species with unusually restricted breeding sites.	Site dependency for dugong reproduction is unknown. However, anecdotal reports suggest dugongs venture into shallow water to reproduce. Therefore, they are highly susceptible to anthropogenic impacts.	Likely to meet this criteria
Species that are very large, long-lived and/or of low fecundity.	The average size of a dugong is 2.4 m, they live to approximately 70 years, bear a single calf, and their generation time is 30 years. Therefore, they are very large, long lived and have low fecundity.	Highly applicable
Species subject to large-scale mass mortality. Catastrophic declines in abundances over a short period.	Mass mortality of dugongs from Hervey Bay in the years 1992 following seagrass destruction from extreme weather conditions (Preen & Marsh, 1995).	Highly applicable
Species subject to prolonged recruitment failure.	Marsh (1995) estimated that even with the most optimistic combination of life history parameters and a low rate of mortality, a dugong population would not increase at more than 6% per year.	Applicable
Species susceptible to environmental stresses.	Dugongs avoid water temperatures less than 16°C (Preen, 1993).	Applicable
Species that are extreme habitat specialists.	Dugongs are dependent on particular seagrasses and the coastal areas in which they grow.	Highly applicable
Obligate supratidal, intertidal, estuarine and coastal embayment species. The susceptibility to human disturbances make these vulnerable.	The dugong inhabits coasts and coastal embayments.	Highly applicable
Species subject to excessive exploitation.	Dugongs continue to be hunted by Indigenous peoples, and often illegally without permit which is difficult to police.	Highly applicable

was scored against the ranking system of Millsap *et al.* (1990) which indicates the degree of biological vulnerability and corresponding action, for species at a national scale (table 12.2). The biological score of 30.6 obtained for the dugong, is comparable to a similar assessment made for the Florida manatee (biological score of 32), ranking them both in the category of 'taxa with biological scores ≥ median for threatened taxa' (Millsap *et al.*, 1990). The dugong was also assessed according to the criteria listed by Jones & Kaly (1995) which highlight potentially vulnerable marine species (table 12.3). In addition to its commodity and cultural values to Indigenous peoples, these assessments (tables 12.2 & 12.3) indicate that the dugong deserves specific conservation status at national and/or regional scales in view of: its specialised diet, long lifespan, low fecundity, coastal distribution, dependence on shallow water calving sites, and taxonomic status as the only extant member of a family and one of only four members of a mammalian order.

Knowledge of the life history, particularly the generation time, of a species enables predictions of the rate of decline or recovery. Detecting a decline in dugong populations is really only possible from a time series of aerial surveys (H.D. Marsh, personal communication). Using this information in conjunction with phylogeography and the predicted rates of interaction among populations is certainly more informative about the status of a species than genetic diversity alone. However, genetic information from historical events (i.e., phylogeography) is particularly relevant to management as the basis for predicting interactions among populations as discussed further below.

12.4 EVALUATION OF THE STATUS OF THE DUGONG AT A GLOBAL SCALE

The dugong is listed by the IUCN (1996) as 'vulnerable to extinction'. Recent anecdotal evidence indicates a decline in the abundance and range of coastal marine mammals, including the dugong, throughout southeast Asia (UNEP, 1996).

The high intraspecific genetic diversity observed for the dugong suggests that it is in 'good genetic health' (table 12.1), that is, the genetic status indicates a low extinction risk. However, even though low genetic diversity

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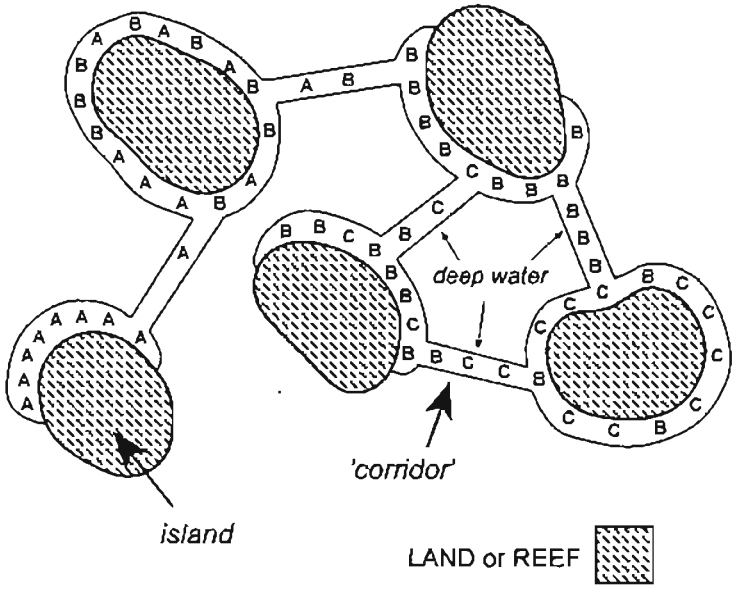
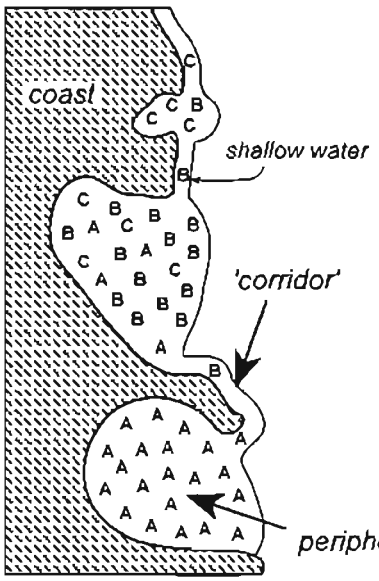
An archipelagic distribution provides more opportunities for the exchange of individuals between populations. Therefore, haplotypes are expected to be more evenly distributed among populations.





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A linear continuous distribution has fewer sources for gene types compared to the archipelagic model. The distribution of haplotypes is suggestive of an isolation-by-distance model (Wright, 1943; 1951; Slatkin, 1993), with populations at the end of the range characterised by lower genetic diversity (White, 1978; Hoffman & Blows, 1994).

Figure 12.1: Schematic representation of the type of distribution for dugong populations and their predicted mode of interaction. Dugong movement along coastlines (as in Australia and West Indian Ocean) may be more frequent than open sea crossings (as in Asia, or between the coast and offshore reefs), therefore the distribution of mtDNA haplotypes (represented by capital letters) may be expected to be more extensive for coastal linear distributions. On the other hand, in an archipelagic distribution the number of source populations for gene flow would be higher and the extent of mtDNA haplotypes more extensive for this reason. Note that this diagram is speculative for the dugong because of the small sample sizes.

can alert the researcher to populations that have undergone historical bottleneck events, genetic diversity *per se*, is not a reliable predictor of genetic health. Conservation decisions are better informed by phylogeography.

Table 12.4: The various intraspecific dugong units. Populations refer to the sample locality. Using the strict definition of Evolutionarily Significant Units (ESUs) by Moritz, (1994a; 1994b) and Moritz *et al.* (1995), there is only one ESU among all of the dugong haplotypes. Haplotype clusters are as illustrated in figure 7.4. Geographic units defined in Chapter 7 are classed into the 'stock categories' outlined by Dizon *et al.* (1992). The recommended Management Units (MUs) refer to the dugongs inhabiting that specific geographic region, and are to be considered independently in a management regime (i.e., an MU would effectively equate to a Dugong Protection Area, DPA). Dugongs of Asia and the West Indian Ocean will have their management status governed by the countries they inhabit. The sample sizes from Asia and the West Indian Ocean are too small for any recommendation based on genetic uniqueness.

Haplotype Clusters	*Geographic units*	*Stock Category*	*Management Units*	*Populations*
Mainly the West Australian haplotype cluster.	North & West Australian	II	The Torres Strait. Other MUs are likely to exist west of the Torres Strait along the Australian coast.	Exmouth Gulf, Broome, Darwin, Borroloola, Boigu Is., Saibai, Warrior Reef, Badu Is., Friday Is.
A combination of the West and East Australian haplotype clusters.	Great Barrier Reef	II	A series of overlapping management units, in which each represents a local population.	Lockhart River, Starcke River, Cairns, Townsville, Bowen, Shoalwater Bay, Rockhampton
Mainly the East Australian haplotype cluster.	South East Queensland	II	Hervey Bay and Moreton Bay.	Hervey Bay, Moreton Bay.
Asian haplotype cluster.	Asian	I	Determined by the local jurisdiction.	Taytay, Ambon, Ranong, Surat Thani.
East Australian haplotype cluster and West Indian Ocean.	West Indian Ocean	III	Determined by the local jurisdiction.	Arabian Gulf, Kenya.

This study indicates that the dugong exists as one Evolutionarily Significant Unit (ESU) (figure 7.4; table 12.4). Within that ESU, three haplotype clusters are evident: West Australian, East Australian and Asian. The available data suggest that haplotypes of the Asian dugongs are distinct from (figure 7.4)

and at least as diverse as (table 7.5) haplotypes of dugongs elsewhere in their range. This pattern may be explained by the spatial arrangement of the dugong populations. Southeast Asian dugongs have an archipelagic distribution compared with the essentially continuous, linear and coastal distribution characteristic of Australian and West Indian Ocean dugongs (figure 12.1). As a consequence, dugong populations in southeast Asia, theoretically should have more source populations for gene flow compared with populations distributed in a linear fashion along continental coastlines (figure 12.1).

Nonetheless, in the event of the regional extinction of Asian dugong populations, the genetic data suggest that it is unlikely that it would be recolonised by dugongs from Australia in a time frame useful to management. The geographic isolation of West Indian Ocean populations also suggests that recolonisation from Asian or Australian dugongs would be unlikely.

The IUCN acknowledges the need for additional guidelines for the application of its global criteria at national or regional scales, in situations where the population of a species within a country is part of a larger (global) population (Gärdenfors, 1996). For example, if a marginal population is not self-sustaining, and depends on even a low level of immigration from the more central parts of its geographic range, the extinction risk within the country/region could be higher than is suggested by the IUCN categories of threat. In this situation, the IUCN criteria are not relevant (Gärdenfors, 1996), which is why regional evaluation is so important for the dugong.

12.5 EVALUATION OF THE STATUS OF THE DUGONG IN THE SOUTHERN GREAT BARRIER REEF

Gärdenfors (1996) advises the conservation manager to consider the following for management issues at the regional scale:

- How abundant is the species in neighbouring regions?
- Are the populations in those regions stable, increasing or decreasing?
- Are there any important threats to those populations?
- If the species becomes extinct in the region, what are the probabilities for recolonisation within 100 years?

In Australia, dugongs have an extensive range, occur in relatively high numbers (more than 85,000), and are genetically diverse (in good genetic health). The recent confirmation of a decline in the southern GBR, has led some scientists to claim that it is 'critically endangered' in this region (Marsh *et al.*, 1996). Is this conclusion legitimate, and if so, what are the implications to management?

The IUCN defined a species as 'critically endangered' when it faces an extremely high risk of extinction in the wild in the immediate future as predicted by at least one of the following:

- A population reduction of 80% over the last 10 years or three generations, whichever is the longer. For dugongs, this period of time is 90 years.
- An area of occurrence of less than 100 km^2 or occupancy less than 10 km^2 (i.e., a severely fragmented range).
- The population size of adults is less than 250 individuals and is also declining.
- The population size of mature individuals is less than 50.
- The probability of extinction is shown by quantitative analysis to be at least 50% within 10 years or three generations, whichever is the longer.

Anecdotal evidence (Bertram & Bertram, 1973; H.D. Marsh, personal communication) and recent documented decline of dugongs (Marsh *et al.*, 1996) suggest that in the southern Great Barrier Reef, their numbers have probably declined more than 80% over the last 90 years. I consider below, the status of the dugong in the southern GBR by addressing the questions posed by Gärdenfors (1996) for conservation managers contemplating the use of the IUCN criteria at a regional scale, in light of the genetic findings of this study.

- **How abundant is the species in neighbouring regions?**

The southern GBR encompasses the dugong populations south of Cape Bedford (17°58'S) to the southern boundary of the Great Barrier Reef Marine Park (24°30'S). The total number of dugongs in the southern GBR is estimated to be 1,642 (s.e. ±236) (table 2.3; Marsh *et al.*, 1996). In the neighbouring populations, the number of dugongs north of Cape Bedford is estimated to be 9,444 (s.e. ±1,381) (Marsh & Corkeron, 1997), and the populations south of the southern GBR: 807 (s.e. ±151) in Hervey Bay and

850 (total count) and Moreton Bay (table 2.3; Marsh *et al.*, 1996; Lanyon & Morrice, 1997). These numbers are likely to be conservative, despite attempts at correcting for dugongs which were not seen because of water turbidity (Marsh & Sinclair, 1989).

- **Are the populations in those regions stable, increasing or decreasing?**

Marsh & Corkeron (1997) indicated that there were no significant differences in dugong densities in the northern GBR from 1984 to 1995, suggesting that dugong numbers are being maintained in this region. The Hervey Bay dugong population, on the other hand, has been severely reduced from an estimated 1,753 (s.e. ±388) in 1988, to 600 (s.e. ±126) in December 1993 following destruction of the seagrass beds from severe weather conditions (Preen & Marsh, 1995). Although there are indications that dugong numbers in Moreton Bay have increased (Lanyon & Morrice, 1997), it is impossible to confirm this due to large temporal variation in the census techniques (H.D. Marsh, personal communication). The April 1993 aerial survey of Moreton Bay (Preen, 1993; Preen & Marsh, 1995) indicated a total count of 664 dugongs, which is higher than any previous counts during the late 1980s (Preen, 1993). A plausible explanation for the increase, is that Moreton Bay has acted as a buffer habitat for the dugongs of Hervey Bay, which left the Bay following the loss of more than 1000 km^2 of habitat in early 1992 (Preen & Marsh, 1995). The suggestion that Hervey and Moreton Bays have exchanged individuals is not contradicted by the genetic data.

- **Are there any important threats to those populations?**

The main threat to dugongs in the northern GBR is Indigenous hunting in a few communities, which are likely to have a local rather than a regional impact (Marsh & Corkeron, 1997). Protection of habitat from pollution, dredging, trawling, terrestrial runoff and coastal development and boat traffic are the main concerns when considering threats to the dugong numbers of Hervey Bay and Moreton Bays (Lanyon & Morrice, 1997).

- **If the species becomes extinct in the region, what are the probabilities for recolonisation within 100 years?**

In genetic terms, the level of gene flow between neighbouring populations in Australia is moderate (table 8.5). This is of conservation importance. If there were no gene flow (i.e., $Nm = 0$), then further subdivision would be of no great concern. However, if there were massive gene flow

(e.g., $Nm = 200$), then it would probably be unlikely for differentiation to occur, regardless of the threats to connectedness of the populations (i.e., fragmentation is unlikely to be a conservation issue from a genetic perspective). Ecologically, the gene flow is very low, and this has implications to recolonisation.

The presence of 'ecologically low' gene flow is supported by: 1) the persistence of the two Australian haplotype clusters despite the Torres Strait being open for the last 200 dugong generations, 2) the presence of high genetic structuring among dugong populations along the east Australian coast (Chapter 7), and 3) the suggested low level of female migration (Nm) (section 8.5), or 'moderate to low' gene flow, between neighbouring populations. There are about three dugong generations in 100 years. Considering the above, it seems unlikely that full recolonisation of a depleted area could occur in that duration.

Furthermore, the level of genetic structuring along the east Australian coastline argues against the hypothesis that the decline of numbers in the southern GBR is due to emigration to other, more northern dugong populations. The genetic evidence supports the conclusion that the decline in the southern GBR is more likely to be due to unsustainable dugong mortality rather than emigration.

12.6 EVALUATION OF THE STATUS OF THE DUGONG IN HERVEY & MORETON BAYS

The Hervey and Moreton Bay dugong populations are at the end of the dugong's range on Australia's southeast coast (figure 3.2), and apparently have lower genetic diversity than the other dugong populations that I examined (table 7.5).

One explanation for the relatively low genetic diversity of Hervey and Moreton Bay dugongs is a population bottleneck resulting from past overexploitation. Dugongs were harvested as an economic resource in Hervey and Moreton Bay for about a century up to the 1960's (section 2.6.7). However, it is unlikely that hunting pressure was extreme enough to cause a genetic bottleneck (see Amos, 1996).

Populations at the margins of a species' range may have lower genetic diversity for reasons other than bottlenecks. Firstly, the Moreton Bay dugong population as a peripheral population, has only one neighbour - Hervey Bay. That is, there is only one source population of immigrating individuals with new genetic types (e.g., figure 12.1). This primary interaction may be reinforced by the likelihood that dugongs move with major currents. For example, after the loss of seagrass in Hervey Bay in early 1992 (Preen & Marsh, 1995), emaciated dugongs were reported south of the dugong's range in line with the warm East Australian Current (figure 11.5). In contrast, few displaced dugongs were found north of Hervey Bay (Preen & Marsh, 1995). This conclusion about current-mediated movement is influenced by the increased chance of carcass recovery south of Hervey Bay because of the higher human population density, and must be regarded as tentative.

Secondly, dugongs from subtropical Moreton and Hervey Bays (figure 3.2), have greater exposure to the environmental thresholds that govern the species' range than more tropical dugong populations. It is likely that as a peripheral population, dugongs in Moreton Bay are continually exposed to environmental stresses, and as a consequence, subjected to severe contractions in population size more frequently than the more tropical populations. Frequent contraction of peripheral populations may be one of the reasons why they tend to have lower genetic diversity. The tendency of a peripheral population (as a marginal population) to be characterised by lower genetic variation was explored by White (1978), and the abiotic and biotic factors and traits leading to a population's being classified as 'marginal' are reviewed by Hoffman & Blows (1994). Climate, resource use, and predation/parasite pressure, are all casual factors determining the distribution of a species, and also determine whether a population will behave as a 'margin'. Marginal populations can be recognised by their genetic and physiological (body condition, fecundity, fertility) fitness (Hoffman & Blows, 1994). Unfortunately, we have no comparative data on the physiological fitness of Moreton Bay dugongs relative to other populations.

It would be interesting to compare the genetic status of the dugong population in Shark Bay at the western end of the dugong's range in Australia, with that of Moreton Bay. As a peripheral population, dugongs of Shark Bay may also be characterised by low genetic variability. However,

Shark Bay does differ from Moreton Bay as a peripheral area in that it harbours a much larger population (in the 1000's rather than 100's), it has been exposed to fewer anthropogenic impacts (from development and hunting pressure), and it provides a larger habitat. It is also likely that Shark Bay was a more significant refuge than Moreton Bay during glaciations as the shelf area was larger (see figure 11.2).

12.7 WHAT IS THE APPROPRIATE MANAGEMENT ACTION TO PROTECT DUGONGS IN EAST QUEENSLAND?

Development of a management regime for the dugong is a complex issue. It is important to assess dugong abundance and any changes in their numbers, and to identify and protect habitats and ensure protection of 'genetically critical populations' (Baker *et al.*, 1994). This study contributes to developing a management regime for the dugong by recognising genetically distinct intraspecific units (table 12.4), and their degree of interaction (historical or contemporary).

Comparison of the mtDNA haplotypes has indicated substructure and distinct geographic units along the Australian coastline. However, the mtDNA data cannot resolve the boundaries of each geographic unit. In addition, given the large and apparently idiosyncratic home ranges of individual dugongs (Marsh & Rathbun, 1990; A.R. Preen, personal communication; figure 2.2), it is impractical to make reserves large enough to contain the home range of every dugong. Therefore, to maximise dugong protection, an approach that uses home range and probability of occurrence, is required in order to reduce the risk of impact to the Australian population. I recommend a series of coastal reserves which encompass the prime dugong habitats, so that most of the dugongs are protected most of the time.

Defining the precise margins of Management Units (or 'Dugong Protection Areas') is best achieved through consideration of dugong distribution and abundance via aerial survey and satellite tracking studies of individual movements (section 2.2.1). Aerial survey provides information on the dugong's range, and identifies areas used by high densities of dugongs. Tracking individual dugongs provides finer scale information on habitat use. Furthermore, the tracking of independent dugong movements

potentially provides information regarding the transit areas (i.e., 'corridors') between populations. However, this is limited as satellite transmitters are pulled underwater when the dugongs travel between feeding areas, so these transit areas are not tracked using the present technology (Marsh & Rathbun, 1990)

The precautionary principle suggests that dugong transit zones between populations as well as major habitats should be protected. This approach would entail declaring virtually the entire east coast of Queensland as a Dugong Protection Area. Although ideal from a conservation perspective, this action is not currently acceptable to the fishing industry which has considerable political clout.

If they exist, areas/routes frequented between populations should be identified and dugong mortality minimised by seasonal prohibition of netting, or speed limits on motorboat travel. As information on dugong transit areas between populations is lacking, a priority in developing a management regime, should be to define areas which support large numbers of dugongs. Such an approach has recently been implemented in the Great Barrier Reef region. In August 1997, the GBR Ministerial Council established a chain of dugong sanctuaries (Dugong Protection Areas, or DPA's) in the southern Great Barrier Reef in response to a serious decline in dugong numbers along 2000 km of the east Queensland coast (Marsh, 1997). In the GBR, gill netting is banned in six Dugong Protection Areas (DPA's) comprising 2395 km^2 of 'A-zoned' areas which support an estimated 55% of the dugongs in the southern GBR. A further 13% of dugongs are in DPA's (B-zones) in which gill netting practices have been modified. Gill netting has also been modified in Hervey Bay with a view to reducing mortality (Marsh, 1997).

By implementing DPA's in the southern GBR, Australia has set a precedent for other marine management regimes in Australia and other countries. If the time series of aerial surveys continues for long enough, we will be able to measure if the implemented DPA's have been effective. However, as pointed out by Marsh (1995), this may take decades.

12.8 IMPLICATIONS OF THIS STUDY FOR OTHER COASTAL SPECIES WITH LARGE RANGES

When the habitats of species with large area requirements such as the dugong are protected, many other species, particularly other large marine animals, are also protected. Therefore, protecting habitat for the dugong means that it acts as an 'umbrella species' (Noss, 1990; Jones & Kaly, 1995). Other coastal marine mammals of the Indo-West Pacific that would benefit from incentives to conserve the dugong at a global scale include: the finless porpoise (*Neophocaena phocaenoides*), Irrawaddy river dolphin (*Orcaella brevirostris*), Indo-Pacific humpback dolphin (*Sousa chinensis*), and the bottlenose dolphin (*Tursiops truncatus*). These species are listed by the IUCN (1996) as 'data deficient', with the exception of the Yangtze River population of the finless porpoise which is 'endangered'.

Within Australian waters, if the dugong were provided with special conservation status, as an umbrella species it would protect coastal areas inhabited by the 'endangered' (IUCN, 1996) marine turtles: Green turtle (*Chelonia mydas*) and Loggerhead turtle (*Caretta caretta*); and the 'data deficient' (IUCN, 1996) dolphins: Irrawaddy dolphin (*Orcaella brevirostris*), bottlenosed dolphin (*Tursiops truncatus*), and the Indo-Pacific humpback dolphin (*Sousa chinensis*).

12.9 FUTURE RESEARCH

Future research should include attempts to gain further insights into female mediated gene flow, to develop an understanding of male mediated gene flow, and to gain an insight into social structure. Addressing these issues would make possible further understanding of dugong ecology, behaviour and phylogeography, which in turn would optimise their conservation management.

12.9.1 Tissue bank

Considering the time and effort invested in establishing a tissue bank for this genetic study, it would of great benefit to future research if the sample collection network continues to develop. Given that I obtained genetic information from dugongs from practically their entire Australian range, I

Table 12.5: Proposed sample collection for future research questions. Larger sample sizes for all dugong populations are required. The 'number to collect' indicates the samples required from various dugong populations (locations) to address the specific research questions. The present sample size refers to samples that have already been collected, of which some have been sequenced (seq.) in this study, and others have not (unseq.).

Dugong population	*Present sample size*		*Number to collect* [a]	*Research Question*
	Seq.	Unseq.		
Australia (north & west coast)				
Shark Bay		1	19	Are peripheral populations characterised by lower genetic diversity?
Exmouth Bay	2	4	14	Does additional genetic subdivision exist for the dugong along the Australian coast west of the Torres Strait?
Port Hedland		4	16	
Roebuck Bay	1	2	17	
Beagle Bay	3	1	16	
Darwin	1	1	18	
Borroloola	1		19	
Australia (east coast)				
Lockhart River	3		17	Can more than three geographic units for the dugong be recognised along the east Australian coast?
Starcke River	6	3	11	
Cairns	2	1	17	
Townsville	9	4	13	
Shoalwater Bay	15		5	
Hervey Bay	6	3	11	
West Indian Ocean				
Arabian coast	3	7	10	Genetic population structure for the dugongs of West Indian Ocean.
Madagascar	1		19	
Mozambique	1		19	
Kenya	1		19	
Asia				
Thailand	4	8	8	Genetic population structure for the dugongs of Asia.
Philippines	1		19	
Indonesia	3	2	15	
India	unsampled		20	Indian dugong phylogeography

[a]Assuming an 'ideal' sample size of 20 per population.

do not expect more than two major haplotype clusters (genetic units) to exist among Australian dugongs. However, I do expect that additional genetic subdivision may be recognised, particularly among dugong populations of the north and west Australian coastline. Obtaining representatives for

those Australian populations presently underrepresented in sample size, would enable this hypothesis to be tested. In addition, further sampling of the east Australian dugong populations may improve the definition of the boundaries of the geographic units presented in Chapter 7. To allow questions of historical phylogeography to be better answered, a larger sample size representing the West Indian Ocean and Asian dugong populations is desirable. An increase in sample size to at least 20 representatives (see section 3.9 for discussion of ideal sample sizes) from each particular dugong population as summarised in table 12.5, would enable the following research questions to be addressed:

- Are populations at the edge of the dugong's range characterised by lower genetic diversity?
- Does additional substructure for the dugong exist along the Australian coast, west of the Torres Strait?
- Can more than three geographic units be recognised among dugong populations of the east Australian coast?
- What is the genetic population structure for the dugongs of the West Indian Ocean?
- What is the genetic population structure for the dugongs of Asia?
- How do the dugongs inhabiting waters of India relate to dugongs elsewhere in the world?

12.9.2 Sex identification using genetics

The gender of a dugong can be identified by examining the proximity of the genital and anal slits, and by inspecting the teats in the axilla (for both see Appendix D for diagram). However, it is sometimes not possible to record gender (e.g., carcass was not intact, or the sample was obtained from a free ranging dugong), or the collector did not record the necessary data (e.g., did not know how to sex a dugong). As a result, the sex of each dugong was not always recorded in this study.

It is possible to determine the sex of each specimen using a genetic marker. Several genetic approaches are available to identify the gender of individuals including cytology (Winn *et al.*, 1973; Lambertsen *et al.*, 1988), and RFLP analysis (Baker *et al.*, 1994). However, the use of the polymerase chain reaction (PCR) to amplify sex chromosome specific regions would be preferred, as dugong samples are not often fresh or well preserved enough

for sexing cytologically or by genomic RFLP analysis. Primers for sex typing using the PCR are reported for birds (Griffiths *et al.*, 1992) and mammals (Palsbøll *et al.*, 1992; Griffiths & Tiwari, 1993; Berube & Palsbøll, 1996). Often the same primers can be used to determine the sex of various species (e.g., sex determining region Y gene (SRY) (Griffiths & Tiwari, 1993)). A research group at the University of Melbourne under the supervision of Professor Marilyn Renfree, is investigating genetic sex identification in the dugong (personal communication).

Haplotype frequencies may be used to detect recent gender-specific movements between populations by dugongs (e.g., as shown by Schaeff *et al.* (1993) for right whales (*Eubalaena glacialis*)). From the available data on gender and haplotypes, there is no indication that male dugongs move to other populations to breed (Appendix C). If the gender of each individual in this study were identified, it might be possible to make predictions of gender specific movements. However, because inferences from haplotype frequencies would require large sample sizes (e.g., about 50 of each gender; e.g., Schaeff *et al.*, 1993), the question of gender specific movements for the dugong would be better addressed with the use of a nuclear marker. Questions regarding social structure would benefit from identification of the gender of each dugong sampled.

12.9.3 Microsatellites

12.9.3.1 Nuclear marker for a population level study

As mtDNA is maternally inherited, the observed phylogenetic pattern from this study indicates that female dugongs are predominantly philopatric, with a small level of permanent migration (<1%) estimated to occur between neighbouring populations (section 8.5). It is possible that male dugongs undergo similar movements. However, satellite tracking of individual dugongs suggests that females may be making greater long distance movements than males, although the statistical significance of this trend is not yet established (A.R. Preen, personal communication) (section 2.4.2; figure 2.2).

As a collaboration with a research group in Florida (laboratories of Peter McGuire & Brian Bowen), the dugong primer sequences I developed (Chapter 9) are being investigated to determine whether they are

informative for manatees. A number of microsatellite markers have been developed for the West Indian manatee and are currently being trialed to examine their phylogeography (A. Garcia-Rodriguez, personal communication). It is possible that these markers may also prove to be useful to re-examine the phylogeographic structure of the dugong.

Questions specifically concerning male-mediated gene flow in the dugong can only be addressed with a nuclear genetic marker, which is why microsatellites were investigated in this study (Chapter 9). An example where a nuclear marker contrasted sharply with the pattern provided by a mitochondrial (maternal) marker within a species, is a study on the green sea turtle (*Chelonia mydas*). A nuclear marker (scnDNA) suggested that interpopulation gene flow for the green turtle was male mediated, whereas the mtDNA marker indicated that females had a tendency for strong natal philopatry (Bowen *et al.*, 1992; Karl *et al.*, 1992). One explanation for this pattern was that the turtle matings take place away from natal sites. As interpopulational exchange of nuclear genes is a major mechanism for maintaining genetic diversity, recognition of sex-specific life histories should be considered in development of a conservation management regime.

12.9.3.2 Social structure

The behaviour of dugongs is difficult to observe. As a result not much is known about their social structure. As more than one individual can share a haplotype, the HVR-1 of mtDNA is not sufficiently diverse as a genetic marker to identify parentage and kinship for the dugong. Employing a nuclear genetic marker such as microsatellites (Chapter 9), in conjunction with genetic sex identification (preceding section), could reveal much information regarding the social structure of the dugong.

Although there is information on the sex and age structure of dugong populations (e.g., Marsh, 1980; Marsh *et al.*, 1984a; 1984b; 1984c), similar information for individual herds is lacking (Preen, 1993). Specific sampling of a dugong herd (i.e., feeding aggregation) with a genetic marker such as microsatellites, may be able to answer questions such as whether herds of dugongs are temporary feeding aggregations or genetically related groups.

12.10 CONCLUSION

The primary objective of this study was to recognise whether intraspecific genetic subdivision existed for the dugong. By obtaining mtDNA sequence information, I have found genetic substructure among Australian dugong populations. There are two Australian genetic types (haplotype clusters), West Australian and East Australian, which overlap in the region of the Great Barrier Reef. Furthermore, dugongs of Australia are genetically distinct from those of Asia. Dugongs have a high genetic diversity and a suggested low level of migration between populations. The suggested philopatry of female dugongs raises concern regarding the rate at which dugongs would repopulate an area after a local mass mortality or extinction. To maintain the high genetic diversity of the dugong, management initiatives should be directed at limiting further fragmentation of their range.

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Contributors of samples. Contact details may not be current, but are the most recent on my record. This appendix continues for an additional 2 pages.

KEY
A Biopsy sampling
B Dugong faecal samples
C Necropsies of dugongs
D Defleshing of skulls
E Collection of tissue samples from Western Australia
F Collection of tissue samples from the east coast of Australia
G Collection of tissue samples from the Torres Strait
H Collection of tissue samples from Asia
I Collection of tissue samples from Africa
J Collection of dugong samples
K Florida manatee (*Trichechus manatus*) sample

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Sirenian tissue bank. Abbreviations include: longitude (LONG), latitude (LAT), sample identification name (ID), haplotype number as listed in table 7.2 (H#), formalin (form), 20% dimethylsulphoxide in saturated sodium chloride (DMSO), 70 to 100% ethyl alcohol (EtOH), male (M), female (F), juvenile (j), M (M), S (S), fresh (fr.) and de. (de.). This appendix continues for an additional 3 pages.

Details of the numbers bracketed throughout the database:

(1) This sequence was not included in the analysis as the result was obtained late in the progress of this study.

(2) The year the animal was captured.

(3) Not included in the analysis because the result was obtained late in the progress of this study. However, see Appendix G.

(4) Potentially a North Queensland dugong. This sample was considered in *Case Study 2* , Chapter 10.

(5) Potentially the same individual. These samples were considered in *Case Study 1* , Chapter 10.

REGION	LONG	LAT	COLLECTED BY	ID	TISSUE SOURCE	SEX	DATE	FLUKE	BODY	FIXER	H#
Trichechus			M.C. Milinkovitch	?	mtDNA	?	?	?	?	lyophilised	no
manatus			Scott Wright	MEC9401	liver	?	?	?	?	DMSO	1
			Scott Wright	MSW9410	kidney, liver	?	?	?	?	?	no
			Scott Wright	MSW9406	kidney	?	?	?	?	?	no
Dugong dugon											
ASIA											
PHILIPPINES											
Philippines			AA (Toba Aquarium)	AA	S	?	1995	?	?	DMSO	no
Taytay	10.47N	119.32E	Lem Aragones	LEM1	salted dried meat	M	??.03.93	adult	adult	DMSO	21
Romblon Is.	12.33N	122.17E	Lem Aragones	LEM2	S	M	??.04.93	?	110	DMSO	38
THAILAND											
Rayong	12.38N	101.17E	K. Adulyanukosol	DU-030	fr. S & M	F	1997	adult	adult	? - DMSO	no
Rayong			K. Adulyanukosol	DU-030c	fr. tissue	M	1997	calf	calf	? - DMSO	no
Rayong			K. Adulyanukosol	DU-065	fr. M	M	1997	?	?	? - DMSO	no
Ranong	9.58N	98.35E	P.Boonyanate	A1	S	F	28.06.93	?	124	buff. EDTA	28
Ranong			K. Adulyanukosol	DU-043	fr. M	F	1997	?	?	? - DMSO	no
Ranong			K. Adulyanukosol	DU-048	de. M	F	1997	?	?	? - DMSO	yes(1)
Surat Thani	9.09N	99.20E	P.Boonyanate	A2	S	M	03.06.93	?	?	dried	27
Surat Thani			P.Boonyanate	A3		?	1993	?	?	?	27
Surat Thani			K. Adulyanukosol	DU-015	fr. S & M	M	1997	?	?	? & DMSO	no
Phang-nga	8.29N	98.31E	K. Adulyanukosol	DU-025	fat	F	1997	?	?	? & DMSO	no
Phang-nga			K. Adulyanukosol	DU-029	fr. S & intestine	M	1997	?	?	? & DMSO	no
Phang-nga			K. Adulyanukosol	DU-031	fat	F	1997	?	?	? & DMSO	no
Phang-nga			K. Adulyanukosol	DU-035	de. M	M	1997	?	?	? & DMSO	no
Phang-nga			K. Adulyanukosol	DU-047	de. S & M	M	1997	?	?	? & DMSO	no
Krabi	8.04N	98.52E	K. Adulyanukosol	DU-028	de. M	M	1997	?	?	? & DMSO	no
Krabi			K. Adulyanukosol	DU-040	fr. M	M	1997	?	?	? & DMSO	no
Krabi			K. Adulyanukosol	DU-049	fr. M	?	1997	?	?	? & DMSO	no
Trang	7.35N	99.35E	K. Adulyanukosol	DU-010	S	F	1997	?	?	form&DMSO	no
Trang			K. Adulyanukosol	DU-012	S	?	1997	?	?	form&DMSO	no
Trang			K. Adulyanukosol	DU-023	de. fat	F	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-033	S	F	1997	?	?	form&DMSO	no
Trang			K. Adulyanukosol	DU-034	S	F	1997	adult	adult	form&DMSO	no
Trang			K. Adulyanukosol	DU-034c	fr. S	F	1997	calf	calf	? & DMSO	no
Trang			K. Adulyanukosol	DU-036	fr. S & M	F	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-037	fr. M	F	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-041	S (biopsy)	F	1997	calf	calf	? & DMSO	no
Trang			K. Adulyanukosol	DU-051	de. M	M	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-052	fr. M	M	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-057	de. M	F	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-058	de. M	M	1997	?	?	? & DMSO	no
Trang			K. Adulyanukosol	DU-059	de. M	M	1997	?	?	? & DMSO	no
INDONESIA											
East Java	6.00S	112.00E	Surabaya zoo	EJ1	S (biopsy)	?	1975(2)	157	282	DMSO	no
?			Mark VanderWal	IND1	liver	?	27.05.93	?	?	?	no
Ambon	3.43S	128.12E	Mark VanderWal	MK	liver	F	19.08.93	?	126	DMSO	22
Ambon			Mark VanderWal	IND2	M, liver, ovary	F	14.09.93	?	287	DMSO	no
Ambon			Hans de Iongh	A4	liver	F	11.10.94	?	?	?	22
Jaya Ancol Ocean.			Janet Lanyon	JL1/D1	S, faeces	F	07.03.97	?	200	DMSO	no
Jaya Ancol Ocean.			Janet Lanyon	JL2/?	faeces	F	03.03.97	?	199	DMSO	no
Jaya Ancol Ocean.			Janet Lanyon	JL3/D2	S	?	07.03.97	?	?	DMSO	no
Toba Aquarium			Chris Marshall	?	faeces	F	??.01.95	adult	adult	?	no
Toba Aquarium			Chris Marshall	?	faeces	F	??.01.95	adult	adult	?	no
Toba Aquarium			Chris Marshall	?	faeces	F	??.01.95	adult	adult	?	no
Toba Aquarium			Chris Marshall	?	faeces	F	??.01.95	adult	adult	?	no
Toba Aquarium			Chris Marshall	?	faeces	M	??.01.95	adult	adult	?	no
WEST INDIAN											
OCEAN											
Arabian Gulf	26.00N	52.00E	Vic Cockcroft	V3	S, M	?	??.03.95	?	?	EtOH	35
Arabian Gulf			Vic Cockcroft	V4	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V5	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V6	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V7a	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V7b	S, M	?	??.03.95	?	?	EtOH	35
Arabian Gulf			Vic Cockcroft	V8	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V9	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V10	S, M	?	??.03.95	?	?	EtOH	no
Arabian Gulf			Vic Cockcroft	V11	S, M	?	??.03.95	?	?	EtOH	15
Madagascar	20.00N	45.00E	Vic Cockcroft	V2	bone	?	??.04.94	?	?	dried	no
Mozambique	15.00N	40.44E	Vic Cockcroft	V1	bone	?	??.10.94	?	?	dried	no
Kenya	1.00N	38.00E	Vic Cockcroft	V12	S, M	?	??.11.94	?	?	EtOH	35

REGION	LONG	LAT	COLLECTED BY	ID	TISSUE SOURCE	SEX	DATE	FLUKE	BODY	FIXER	H#
TORRES STRAIT											
(central & western			G. Dews (CSIRO)	TS29	M (hunted)	?	19.05.93	?	?	EtOH	no
group of islands)			G. Dews (CSIRO)	TS30	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS32	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS33	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS34	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS35	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS36	M (hunted)	?	19.05.93	?	?	dried	no
			G. Dews (CSIRO)	TS37	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS38	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS39	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS40	M (hunted)	?	19.05.93	?	?	EtOH	no
			G. Dews (CSIRO)	TS41	M (hunted)	?	19.05.93	?	?	EtOH	no
Friday Island	10.36S	142.10E	John Meany	F1	S, M (hunted)	M?	24.01.93	adult	adult	DMSO	10
North Qld?			Peter Stratton	PS	fr. M (forensic)	?	??.11.96	?	?	frozen	yes(4)
GREAT BARRIER											
REEF											
Lockhart River	12.58S	143.31E	Weipa Police	LH1	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11
Lockhart River			Weipa Police	LH2	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10
Lockhart River			Weipa Police	LH3	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lockhart River			Weipa Police	LH4	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11(5)
Lockhart River			Weipa Police	LH5	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lockhart River			Weipa Police	LH6	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lockhart River			Weipa Police	LH7	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11(5)
Lockhart River			Weipa Police	LH8	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lockhart River			Weipa Police	LH9	S (hunted-forensic)	?	??.11.93	?	?	EtOH	13
Lockhart River			Weipa Police	LH10	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lockhart River			Weipa Police	LH11	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11(5)
Lockhart River			Weipa Police	LH12	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11(5)
Lockhart River			Weipa Police	LH13	S (hunted-forensic)	?	??.11.93	?	?	EtOH	11(5)
Lockhart River			Weipa Police	LH14	S (hunted-forensic)	?	??.11.93	?	?	EtOH	10(5)
Lizard Island	14.40S	145.28E	J. Morgan	LIZ	S (biopsy)	?	1994	?	?	?	no
Starcke River	14.49S	144.58E	Blair & Tikel	H1	S, M (hunted)	M	06.01.93	100	270	EtOH, DMSO	11
Starcke River			John Bowen	HV2	S (hunted)	?	?	?	?	EtOH	11
Starcke River			John Bowen	HV3	S (hunted)	?	?	?	?	DMSO	8
Starcke River			John Bowen	HV4	S (hunted)	?	?	?	?	DMSO	11
Starcke River			John Bowen	HV5	S (hunted)	F	16.08.94	?	?	DMSO	yes
Starcke River			John Bowen	HV6	S (hunted)	F	17.08.94	?	?	DMSO	no
Starcke River			John Bowen	HV7	S (hunted)	F	17.08.94	?	?	DMSO	yes
Starcke River			John Bowen	HV8	S (hunted)	F	17.08.94	?	?	DMSO	no
Starcke River			John Bowen	HV9	S (hunted)	M	16.08.94	?	?	DMSO	no
Cairns	16.51S	145.43E	Karen Vidler	319	de. carcass (head)	M	07.04.93	?	285	frozen	16
Mission Bay	16.53S	145.53E	Geoff Kelly	GBMS	S	F	15.12.93	?	260	DMSO	no
Yarrabah	16.55S	145.52E	Harvey Thomas	Y1	S (hunted)	M	22.04.93	?	?	DMSO	11
Cardwell(Oyst. Pt.)			D.Blair	?	necropsy (planted?)	M	12.12.96	?	?	frozen	no
Townsville	19.16S	146.49E	Jeff Miller & Tikel	313	fr. M (necropsy)	M	08.09.92	90	273	EtOH	9
Townsville			Marsh et al.	315	de. liver, gonad (necr.)	M	17.12.92	?	247	EtOH	9
Townsville			Morgan & Tikel	317	de. various (necropsy)	M	14.01.93	?	240	frozen	15
Townsville			Aragones & Dalton	321	de. ? (necropsy)	M	24.08.93	?	289	frozen	15
Townsville			Morgan et al.	322	de. S (necropsy)	?	06.11.93	?	275	frozen	11
Townsville			Morgan & Tikel	323	de. various (necropsy)	F	12.03.94	100	305	frozen	9
Townsville			Graham & Tikel	324	de. skull (necropsy)	?	17.06.94	90	300	frozen	no
Townsville			Miles & Tikel	325	de. various (necr.)	M	18.07.94	no tail	190+	frozen	no
Townsville			Blair & Morgan	327	rel. fr. various (necr.)	M	01.01.95	?	222	frozen	26
Townsville			Blair & Morgan	328	rel. fr. various (necr.)	M	01.01.95	?	208	frozen	15
Townsville			Tikel et al.	329	fr. various (necropsy)	M	21.04.95	86	275	frozen?	9
Townsville			Preen et al.	330	de. various (necropsy)	M	10.07.95	80	260	frozen?	no
Townsville			Miller & Lap	331	fr. various (necropsy)	M	25.07.95	?	293	frozen?	no
Bowen	20.00S	148.10E	S. Watson & D. Tikel	316	fr. liver, S (necr.)	F	23.12.92	45	210	froz., EtOH	12
Shoalwater Bay	22.22S	150.23E	Col Limpus	SB1	S (biopsy)	F	27.07.94	?	?	DMSO	11
Shoalwater Bay			Col Limpus	SB2	S (biopsy)	?	28.07.94	?	?	DMSO	29
Shoalwater Bay			Col Limpus	SB3	S (biopsy)	?	28.07.94	?	?	DMSO	26
Shoalwater Bay			Col Limpus	SB4	S (biopsy)	?	28.07.94	?	?	DMSO	30
Shoalwater Bay			Col Limpus	SB5	S (biopsy)	?	28.07.94	?	?	DMSO	31
Shoalwater Bay			Col Limpus	SB6	S (biopsy)	?	29.07.94	?	?	DMSO	6
Shoalwater Bay			Col Limpus	SB7	S (biopsy)	?	29.07.94	?	?	DMSO	15
Shoalwater Bay			Col Limpus	SB8	S (biopsy)	?	30.07.94	?	?	DMSO	15
Shoalwater Bay			Col Limpus	SB9	necropsy	M	31.07.94	?	?	DMSO	30
Shoalwater Bay			Col Limpus	SB10	S (biopsy)	?	01.08.94	?	?	DMSO	32
Shoalwater Bay			Col Limpus	SB11	S (biopsy)	?	01.08.94	?	?	DMSO	33
Shoalwater Bay			Col Limpus	SB12	S (biopsy)	?	01.08.94	?	?	DMSO	15
Shoalwater Bay			Col Limpus	SB13	S (biopsy)	?	01.08.94	?	?	DMSO	15
Shoalwater Bay			Col Limpus	SB14	S (biopsy)	?	01.08.94	?	?	DMSO	36
Shoalwater Bay			Col Limpus	SB15	S (biopsy)	?	01.08.94	?	?	DMSO	11
Rockhampton	23.22S	150.32E	King	KING	S	M	21.10.93	?	265	EtOH	9
Rockhampton			Bruce Hill	ROCKY	liver			?	?	frozen	20
Rockhampton?			Lem Aragones	326	? (head)	?	?	?	?	frozen	no

REGION	LONG	LAT	COLLECTED BY	ID	TISSUE SOURCE	SEX	DATE	FLUKE	BODY	FIXER	H#
NORTH & WEST											
AUSTRALIA											
Shark Bay	26.00S	113.30E	R.Prince	WA1	M	?	1991/92	?	?	dried	no
Exmouth Bay	22.20S	114.09E	R.Prince	WA	?	F	02.07.93	adult	adult	?	17
Exmouth Bay			Ric Kamiewicz	RK	S (carcass)	F	25.06.94	?	265	DMSO	14
Exmouth Bay			Prince & Kamiewicz	WA2	M	F	05.01.95	adult	adult	DMSO?	no
Exmouth Bay			Prince & Kamiewicz	EG4	soup	F	01.08.95	?	?	DMSO?	no
Exmouth Bay			Prince & Kamiewicz	WA8	M	?	06.10.95	?	?	DMSO?	no
Exmouth Bay			Prince & Kamiewicz	WA7	S	?	01.11.95	?	?	DMSO	no
Dampier	20.39S	116.43E	Prince & Stanley	WA6	S	F	28.09.95	?	?	DMSO	no
Port Hedland	20.18S	118.35E	Prince & Macintosh	PH1	soup	M	07.09.95	?	?	DMSO	no
Port Hedland			Prince & Macintosh	PH2	soup	M	14.09.95	?	?	DMSO	no
Port Hedland			Prince & Macintosh	PH3	S	M	01.11.95	?	?	DMSO	no
Roebuck Bay	17.58S	122.15E	R.Prince	BM1	M (hunted)	M	06.07.94	?	est.ca 2	DMSO	20
Roebuck Bay			Prince & McCarthy	WA4	?	?	1993	?	?	DMSO	no
Roebuck Bay			R.Prince	BM3	M	M	??.06.95	?	?	DMSO	no
Beagle Bay	16.56S	122.32E	R.Prince	BB0	M (hunted)	Fc	10.07.94	?	?	DMSO	3
Beagle Bay			R.Prince	BB1	M (hunted)	M	01.07.94	?	est. 2	DMSO	34
Beagle Bay			R.Prince	BB2	M (hunted)	M	15.07.94	?	est. 3	DMSO	3
Beagle Bay			R.Prince	BB3	M (hunted)	F	09.07.94	?	?	DMSO	no
Western Australia			R.Prince	WA3	M or fat?	?	1994	?	?	DMSO	no
Western Australia			R.Prince	WA5	liver & kidney?	?	1994	?	?	DMSO	no
Ashmore Reef	12.20S	123.10E	Scott Whiting	SW1	S (bloggy)	?	16.05.97	adult	adult	DMSO	yes(3)
Ashmore Reef			Scott Whiting	SW3	S (bloggy)	?	07.11.97	adult	adult	DMSO	no
Ashmore Reef			Scott Whiting	SW4	S (bloggy)	?	07.11.97	adult	adult	DMSO	no
Darwin	12.23S	130.44E	John Bradley	DAR/DR16	S	F	12.05.94	?	240	DMSO	2
Bare Sand Is.	12.32S	130.25E	Scott Whiting	SW2	S	?	??.07.97	adult	adult	DMSO	no
Borroloola	16.00S	136.15E	L. Aragones & Preen	SmB	faeces (free ranging)	?	?	?	?	frozen	37
Borroloola			L. Aragones & Preen	LgeB	faeces (free ranging)	?	?	?	?	frozen	no
Borroloola			Keith Saalfeld	DR11/KS1	S	F	03.08.95	?	165	DMSO	no
Australian Gulf	15.19S	13609E	Tony Preen	TP1	faeces (free ranging)	?	?	?	?	DMSO	no
Australian Gulf			Tony Preen	TP2	faeces (free ranging)	?	?	?	?	DMSO	no
TORRES STRAIT											
Boigu Is.	9.17S	142.13E	A. Harris (CSIRO)	T673	M (hunted)	M	05.12.91	68	?	EtOH	9
Boigu Is.			A. Harris (CSIRO)	T674	M (hunted)	M	05.12.91	64	?	EtOH	6
Boigu Is.			A. Harris (CSIRO)	T675	M (hunted)	M	05.12.91	63	?	EtOH	6
Boigu Is.			A. Harris (CSIRO)	T676	M (hunted)	F	04.12.91	87	?	EtOH	2
Boigu Is.			G. Dews (CSIRO)	TS26	M (hunted)	M	06.02.93	99	?	EtOH	no
Boigu Is.			G. Dews (CSIRO)	TS3	M (hunted)	F	15.05.92	85	?	EtOH	23
Boigu Is.			G. Dews (CSIRO)	TS4	M (hunted)	F	15.05.92	101	?	EtOH	4
Boigu Is.			G. Dews (CSIRO)	TS5	M (hunted)	F	16.05.92	90	?	EtOH	3
Boigu Is.			G. Dews (CSIRO)	TS6	M (hunted)	F	16.05.92	92	?	EtOH	5
Boigu Is.			G. Dews (CSIRO)	TS8	M (hunted)	M	29.02.92	?	243	EtOH	6
Boigu Is.			G. Dews (CSIRO)	TS9	M (hunted)	M	29.02.92	?	?	EtOH	7
Boigu Is.			G. Dews (CSIRO)	TS10	M (hunted)	M	01.03.92	74	?	EtOH	8
Saibai	9.23S	142.40E	A. Harris (CSIRO)	T677	M (hunted)	F	02.12.91	102	?	EtOH	2
Daru	9.04S	143.21E	CSIRO	TS46	M, S, fat (hunted)	?	22.06.92	?	?	DMSO	no
Daru			CSIRO	TS47	M, S, fat (hunted)	?	25.06.92	?	?	DMSO	no
Daru			CSIRO	TS48	M, S, fat (hunted)	?	26.06.92	?	?	DMSO	no
Daru			CSIRO	TS49	M, S, fat (hunted)	?	02.09.92	?	?	DMSO	no
Daru			CSIRO	TS50	M, S, fat (hunted)	?	01.10.92	?	?	DMSO	no
Daru			CSIRO	TS51	M, S, fat (hunted)	?	13.10.92	?	?	DMSO	no
Daru			CSIRO	TS52	M, S, fat (hunted)	?	16.10.92	?	?	DMSO	no
Warrior Reefs	9.31S	143.07E	A. Harris (CSIRO)	T679	M (hunted)	M	17.11.91	108	?	EtOH	10
Badu Is.	10.07S	142.07E	A. Harris (CSIRO)	T678	M (hunted)	F	23.11.91	78	?	EtOH	2
Badu Is.			G. Dews (CSIRO)	TS7	M (hunted)	M	14.04.92	?	195	EtOH	6
Badu Is.			G. Dews (CSIRO)	TS11	M (hunted)	M	14.04.92	?	195	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS12	M (hunted)	M	04.05.92	74	?	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS13	M (hunted)	F	04.05.92	75	?	EtOH	no
Badu Is.			CSIRO	TS14	S, M, fat (hunted)	F	21.09.92	?	233	EtOH	no
Badu Is.			CSIRO	TS15	M (hunted)	F	21.09.92	73	?	EtOH	no
Badu Is.			CSIRO	TS16	M (hunted)	M	22.09.92	69	?	EtOH	no
Badu Is.			CSIRO	TS17	M (hunted)	?	23.09.92	?	?	EtOH	no
Badu Is.			CSIRO	TS18	M (hunted)	F	28.10.92	?	251	EtOH	no
Badu Is.			CSIRO	TS19	M (hunted)	M	28.10.92	?	257	EtOH	no
Badu Is.			CSIRO	TS20	M (hunted)	M	28.10.92	?	228	EtOH	no
Badu Is.			CSIRO	TS21	M (hunted)	F	30.10.92	53	?	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS22	M (hunted)	?	09.02.93	?	224.5	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS23	M (hunted)	?	09.02.93	?	252	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS24	M (hunted)	?	09.02.93	?	200	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS25	M (hunted)	?	07.02.93	?	263	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS27	M (hunted)	M	15.12.92	?	206	EtOH	no
Badu Is.			G. Dews (CSIRO)	TS28	M (hunted)	M	14.12.92	?	186	EtOH	no
Badu Is.			Peter Massey	TS43	M (hunted)	?	12.01.94	?	?	DMSO	no
Badu Is.			Peter Massey	TS44	M (hunted)	M	12.01.94	?	?	DMSO	6
Badu Is.			Peter Massey	TS45	M (hunted)	M	12.01.94	?	?	DMSO	3
Mabuiag	9.57S	142.10E	G. Dews (CSIRO)	TS1	M (hunted)	F	06.05.92	?	255	EtOH	2
Mabuiag			G. Dews (CSIRO)	TS2	M (hunted)	F	07.05.92	?	257	EtOH	3

REGION	LONG	LAT	COLLECTED BY	ID	TISSUE SOURCE	SEX	DATE	FLUKE	BODY	FIXER	H#
SOUTH EAST											
QUEENSLAND											
Hervey Bay	24.57S	152.40E	Tony Preen	B02/HB18	S	F	21.10.92	?	267	DMSO	15
Hervey Bay			Col Limpus	HB1	S (biopsy)	?	26.05.94	adult	adult	DMSO	24
Hervey Bay			Col Limpus	HB2	S (biopsy)	?	26.05.94	adult	adult	DMSO	15
Hervey Bay			Col Limpus	HB3	S (biopsy)	?	26.05.94	adult	adult	DMSO	19
Hervey Bay			Alan Jeffrey	HB4	S	?	1995	?	?	DMSO	15
Hervey Bay			Alan Jeffrey	HB5	M	?	1994	?	?	DMSO	11
Hervey Bay			Steve Rowe	HB6	S	?	08.09.96	?	260	DMSO	no
Hervey Bay			Steve Rowe	HB7	S	?	09.09.96	?	240	DMSO	no
Hervey Bay			Steve Rowe	HB8	S	?	09.09.96	?	200	DMSO	no
Moreton Bay	27.25S	153.20E	Tikel et al.	D1	S (biopsy)	?	18.05.92	adult	adult	conc. NaCl	6
Moreton Bay			Tikel et al.	D2	S (biopsy)	?	18.05.92	adult	adult	conc. NaCl	15
Moreton Bay			? (UQ)	D3	head	?	18.05.92	adult	adult	frozen	18
Moreton Bay			Slade et al.	D4	S (biopsy)	?	22.07.92	adult	adult	frozen	15
Moreton Bay			Slade et al.	D5	S (biopsy)	?	22.07.92	adult	adult	frozen	no
Moreton Bay			Slade et al.	D6	S (biopsy)	?	22.07.92	adult	adult	frozen	no
Moreton Bay			T. Preen (Uni Qld)	MB6	S	M	14.04.93	adult	adult	DMSO	15
Moreton Bay			Col Limpus	CL1	S (biopsy)	?	29.05.94	adult	adult	DMSO	15
Moreton Bay			Col Limpus	CL2	S (biopsy)	?	29.05.94	adult	adult	DMSO	6
Moreton Bay			Col Limpus	CL3	S (biopsy)	?	29.05.94	adult	adult	DMSO	6
Moreton Bay			Col Limpus	CL4	S (necropsy)	M	08.08.94	?	295	DMSO	no
Moreton Bay			Col Limpus	CL5	S (biopsy)	?	07.10.94	adult	adult	DMSO	no
Moreton Bay			Col Limpus	CL6	S (biopsy)	?	07.10.94	adult	adult	DMSO	15
Moreton Bay			Col Limpus	CL7	S (biopsy)	?	07.10.94	adult	adult	DMSO	15
Moreton Bay			Col Limpus	CL8	S (biopsy)	?	07.10.94	adult	adult	DMSO	no
Moreton Bay			Col Limpus	CL9	S (biopsy)	?	07.10.94	adult	adult	DMSO	no
Moreton Bay			Col Limpus	CL10	S (biopsy)	?	07.10.94	adult	adult	DMSO	no
Moreton Bay			H.Marsh	HEART	heart M	?	27.05.03	?	?	frozen	15
MB(Stradbroke Is.)			Corkeron & Brown	314	de, head (necropsy)	?	??.08.92	adult	adult	frozen	15
MB (Bribie Island)	27.00S	153.10E	?	318	head, poss. sample D3	?	?	?	?	frozen	no
MB (Bribie Island)			?	320	head (necropsy)	?	?	?	?	frozen	15
Ibuka (Shark Bay)	26.25S	153.21E	Philip Gibbs	PGU	fat	?	25.11.92	?	?	?	15
Hastings River	31.25S	152.49E	Philip Gibbs	PGA	fat	F	21.10.92	?	217	fresh	no
Nelson Bay	32.44S	152.08E	Philip Gibbs	PGB	M, liver, S	F	07.10.92	?	233	fresh	25
Newcastle	32.55S	151.46E	Philip Gibbs	ORC120	fat, S	F	11.01.93	?	288	fresh	15
			Philip Gibbs	ORC121	M, fat	M	28.01.93	?	280	fresh	15
SYDNEY	33.55S	151.10E									
Botany Bay			Philip Gibbs	ORC107	M, liver, S	M	10.09.92	?	204	just fresh	18
			Philip Gibbs	PG115	M, liver, S	F	30.10.92	?	317	autolysis	no
Cronulla			Philip Gibbs	ORC139	M, liver, S	F	22.03.94	?	284	just off	11
Canberra			H.Marsh	CAN	liver (+ barnacles)	?	09.09.92	?	?	frozen	no
Jervis Bay	35.03S	150.44E	Philip Gibbs	ORC106	M, S	F	28.08.92	?	282	just fresh	15

Type of tissue. Not much tissue is needed. For example, tissue of the dimensions 1cm x 0.5cm is ample. Any tissue already preserved in the following state would be ideal: frozen tissue (including blood samples), tissue in alcohol or dried tissue (i.e., salted meat). Tissue preserved in formalin is not favoured.

The most favoured tissue from a fresh carcass is gonad, liver or muscle. If the carcass is not fresh, skin (the grey epidermal layer) and/or muscle is the most favoured. The tissue should be preserved immediately. I have provided you with tubes containing 20% DMSO in saturated NaCl solution for this purpose. If the biopsy is to be taken from a live animal, a skin biopsy may be surgically removed using a scalpel blade. All surgical instruments should be sterilised regardless of whether the animal is dead or not.

Surgical removal of a skin biopsy from a live captive animal. I would like to stress that we only need a small amount of tissue, the epidermis of skin (grey in colour) contains the most DNA. The epidermis may be scraped off using a sterile scalpel and is only about 1 - 2mm thick. The white blubber layer immediately beneath the skin is fibrous and is NOT suitable. Once the skin biopsy is obtained, the tissue should be immediately placed into the preservation solution. Please label using the black marker pen on the outside of the sample containers, and use pencil on the waterproof paper to label the sample inside the containers.

Details of the tissue collection would be most appreciated. This includes sex, age, where the dugong was captured and any other details about the animal that is available. Please also include the details of collection of the biopsy, that is, from what part of the body the biopsy was taken.

In the kit that I have provided, you will find:
(1) black marker pen
(2) pencil and waterproof paper labels
(3) 15 scalpel blades
(4) scalpel handle
(5) disposable blade remover
(6) pair of gloves (to protect your hands from the DMSO)
(7) pair of forceps (wash and flame forceps to sterilise)
(8) 10 sample containers with 20% DMSO in saturated NaCl.

Please use **caution with DMSO** as it is toxic and mildly flammable. It does not require declaration for transport on aircraft, as it is not toxic or flammable enough to require a united nations chemical code. However, it is hazardous to your health and should not be ingested. If DMSO comes in contact with your skin it should be washed off with soap and water.

DUGONG CARCASS DATA SHEET

Specimen number:.....

Examined by:..... Contact address:.....

Location:.....

Date of examination:..... Time:..... Estimated time since death:.....

Condition: live / fresh dead / fair / bloated / collapsed

Photos taken? Yes / No

External marks (bites, nets etc):.....

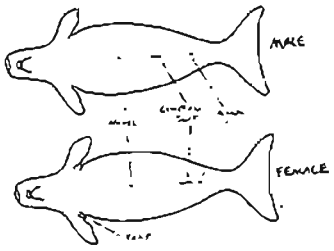
.....

Body length (see illustration):.....m

Length of teats: Left:.....cm Right:.....cm

Gender (see illustration): Male / Female

Tusks present: Yes / No



NOTE: This is a hierarchy of observations proceed as far as you feel competent.

Comments on external features:

Skin.....

Eyes.....

Nostrils.....

Flippers.....

Tail fluke.....

General physical condition: good / poor

Foetus present? Length ..... Gender ..... Weight .....

Milk being produced? (cut mammary gland) .....

Comments on internal organs:

Body-wall fat: firm & white / yellow, jelly-like & watery

Stomach: full / half full / less

Intestines: packed with food / relatively empty

external colour: white / yellow / pink / red / other..

Liver: colour .....

edge profile: rounded / sharp

Respiratory tract:

Trachea: internal colour: white / pink / red / other

Lungs: colour: pink / red / other .....

texture: soft / firm

Heart:

colour of surrounding fat: yellow / white / other

texture of fat: firm / soft / jelly / other

Possible Samples (label fully)

Organ	Preparation	Analyses
Body fat	Freeze	Toxins, H.metals
Blood	Anti-coagulant	Red cell count
	No anti-coagulant	Blood chemistry
Skin	Freeze	Genetics
Stomach content	Freeze/formalin*	Diet, toxic dinoflagellates
Liver	Freeze	Toxins
Gall bladder	Freeze	Toxins
Gonads	Formalin/freeze	Reprod history
Skull	Freeze/deflesh	Age

* 10% seawater formalin (9 parts seawater to 1 part formalin)

Comments:

A mtDNA PAUP phylogeny required a total of 66 steps. The table of steps required was drawn from *character diagnostics* in PAUP under the *Describe Trees* option.

Steps at each site:

	0	1	2	3	4	5	6	7	8	9
0		0	1	0	0	1	0	0	2	0
10	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	1	2	1	2	0	1
30	0	0	0	1	2	3	1	0	2	1
40	0	0	2	2	0	2	0	0	3	0
50	0	1	0	1	0	1	1	0	0	0
60	2	2	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0
90	2	2	0	0	1	0	0	0	2	0
100	0	0	0	0	0	0	0	1	0	0
110	0	0	0	2	0	0	2	0	0	0
120	0	0	3	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0
140	0	0	0	0	0	0	0	0	0	0
150	1	0	0	4	1	0	0	0	0	0
160	0	0	0	0	0	0	0	0	0	0
170	1	0	0	0	0	0	0	0	1	4
180	0	0	0	0	0	0	0	1	0	0
190	0	0	0	0	0					

Summary:

Substitutions per site	Observed frequency
0	155
1	19
2	15
3	3
4	2

MEGA output of pairwise comparisons of haplotypes. Haplotype numbers correspond with those in table 7.1. This table continues for an additional page.

Input Data: DNA
No. of Otus used: 38 of 38

Distance: Gamma distance with Tamura-Nei model, with (a=0.3168)
No. of nucleotides in subset: 196 of 196
Gap sites and missing infomation data: All such sites were removed only in the pairwise comparisons.

OTUs	1	2	3	4	5	6	7	8	9
1									
2	1.3793								
3	1.5756	0.0108							
4	1.0744	0.0728	0.0688						
5	1.4794	0.0054	0.0166	0.0839					
6	1.0044	0.0860	0.0837	0.0117	0.0971				
7	1.3984	0.0108	0.0228	0.0571	0.0166	0.0688			
8	1.0010	0.0789	0.0757	0.0055	0.0900	0.0055	0.0626		
9	1.4760	0.0170	0.0055	0.0626	0.0228	0.0758	0.0166	0.0688	
10	1.0694	0.0939	0.0927	0.0187	0.1050	0.0055	0.0758	0.0117	0.0837
11	1.3770	0.0228	0.0108	0.0533	0.0290	0.0665	0.0228	0.0595	0.0053
12	1.2937	0.0054	0.0166	0.0838	0.0111	0.0971	0.0166	0.0900	0.0228
13	1.3887	0.0108	0.0227	0.0688	0.0166	0.0688	0.0117	0.0626	0.0297
14	1.4766	0.0166	0.0053	0.0595	0.0228	0.0744	0.0290	0.0664	0.0108
15	0.9494	0.0971	0.0939	0.0170	0.1092	0.0053	0.0790	0.0108	0.0860
16	0.9492	0.1092	0.1050	0.0228	0.1225	0.0111	0.0901	0.0166	0.0971
17	1.3639	0.0227	0.0108	0.0664	0.0290	0.0834	0.0360	0.0743	0.0170
18	1.0248	0.0901	0.0860	0.0108	0.1022	0.0108	0.0728	0.0170	0.0790
19	0.8581	0.0971	0.1171	0.0290	0.1092	0.0173	0.0790	0.0228	0.1092
20	1.2937	0.0054	0.0166	0.0626	0.0111	0.0758	0.0166	0.0688	0.0228
21	1.1430	0.0936	0.0746	0.0927	0.1029	0.0757	0.0928	0.0836	0.0657
22	1.1427	0.1241	0.1026	0.1029	0.1363	0.0859	0.1261	0.0938	0.0938
23	1.3725	0.0055	0.0170	0.0789	0.0108	0.0939	0.0170	0.0859	0.0240
24	0.9007	0.1092	0.1304	0.0358	0.1225	0.0241	0.0901	0.0295	0.1225
25	0.9978	0.1029	0.1029	0.0266	0.1140	0.0117	0.0838	0.0186	0.0927
26	1.4760	0.0290	0.0166	0.0447	0.0357	0.0579	0.0166	0.0509	0.0111
27	1.1427	0.1241	0.1026	0.1029	0.1363	0.0859	0.1261	0.0938	0.0938
28	1.0136	0.1035	0.1038	0.0841	0.1147	0.0689	0.1055	0.0759	0.0933
29	0.9491	0.0971	0.0939	0.0170	0.1092	0.0053	0.0790	0.0108	0.0860
30	0.9494	0.0758	0.0744	0.0290	0.0860	0.0173	0.0790	0.0228	0.0665
31	1.4760	0.0290	0.0166	0.0626	0.0357	0.0758	0.0296	0.0688	0.0111
32	0.9007	0.1092	0.1304	0.0358	0.1225	0.0241	0.0901	0.0295	0.1225
33	0.8546	0.1050	0.1261	0.0360	0.1172	0.0228	0.0860	0.0290	0.1171
34	1.4760	0.0054	0.0055	0.0626	0.0111	0.0758	0.0166	0.0688	0.0117
35	1.3840	0.0928	0.0743	0.0580	0.1029	0.0430	0.0745	0.0500	0.0664
36	1.4380	0.0285	0.0163	0.0597	0.0349	0.0733	0.0286	0.0660	0.0107
37	1.4776	0.0111	0.0108	0.0728	0.0174	0.0860	0.0228	0.0789	0.0170
38	0.9656	0.0939	0.0757	0.0580	0.1050	0.0447	0.0971	0.0509	0.0688

OTUs	10	11	12	13	14	15	16	17	18
10									
11	0.0744								
12	0.1050	0.0290							
13	0.0758	0.0360	0.0166						
14	0.0834	0.0166	0.0228	0.0290					
15	0.0108	0.0758	0.1092	0.0790	0.0837				
16	0.0166	0.0860	0.1225	0.0900	0.0939	0.0053			
17	0.0935	0.0227	0.0290	0.0359	0.0055	0.0927	0.1029		
18	0.0170	0.0688	0.1022	0.0860	0.0758	0.0055	0.0108	0.0837	
19	0.0228	0.0971	0.0860	0.0790	0.1050	0.0111	0.0173	0.1140	0.0166
20	0.0837	0.0170	0.0111	0.0166	0.0228	0.0860	0.0971	0.0290	0.0790
21	0.0836	0.0578	0.1029	0.0925	0.0667	0.0859	0.0970	0.0767	0.0939
22	0.0938	0.0836	0.1362	0.1259	0.0925	0.0970	0.1091	0.1025	0.1050
23	0.1029	0.0297	0.0108	0.0170	0.0227	0.1050	0.1171	0.0297	0.0971
24	0.0295	0.1092	0.0971	0.0900	0.1171	0.0173	0.0241	0.1261	0.0228
25	0.0055	0.0834	0.1140	0.0836	0.0935	0.0170	0.0228	0.0832	0.0240
26	0.0659	0.0173	0.0357	0.0297	0.0228	0.0665	0.0758	0.0290	0.0595
27	0.0938	0.0836	0.1362	0.1259	0.0925	0.0970	0.1091	0.1025	0.1050
28	0.0759	0.0840	0.1146	0.1053	0.0944	0.0790	0.0901	0.1063	0.0862
29	0.0108	0.0758	0.1092	0.0789	0.0837	0.0111	0.0173	0.0927	0.0166
30	0.0228	0.0579	0.0860	0.0790	0.0659	0.0111	0.0173	0.0748	0.0166
31	0.0837	0.0053	0.0357	0.0427	0.0228	0.0860	0.0971	0.0290	0.0790
32	0.0295	0.1092	0.0971	0.0900	0.1171	0.0173	0.0241	0.1261	0.0228
33	0.0290	0.1050	0.0939	0.0859	0.1140	0.0166	0.0228	0.1241	0.0228
34	0.0837	0.0170	0.0111	0.0166	0.0108	0.0860	0.0971	0.0170	0.0790
35	0.0500	0.0579	0.1029	0.0743	0.0657	0.0509	0.0595	0.0746	0.0579
36	0.0814	0.0052	0.0349	0.0420	0.0222	0.0828	0.0932	0.0285	0.0756
37	0.0939	0.0228	0.0173	0.0228	0.0166	0.0971	0.1092	0.0227	0.0901
38	0.0509	0.0595	0.1050	0.0970	0.0664	0.0533	0.0626	0.0743	0.0595

OTUs	19	20	21	22	23	24	25	26	27
19									
20	0.0860								
21	0.1091	0.0850							
22	0.1224	0.1130	0.0289						
23	0.1050	0.0108	0.1051	0.1357					
24	0.0053	0.0971	0.1224	0.1370	0.1171				
25	0.0290	0.0927	0.0925	0.1026	0.1130	0.0357			
26	0.0860	0.0357	0.0836	0.0938	0.0360	0.0971	0.0748		
27	0.1224	0.1130	0.0173	0.0116	0.1357	0.1370	0.1026	0.0938	
28	0.1022	0.0933	0.0930	0.0837	0.1140	0.1155	0.0839	0.0933	0.0837
29	0.0241	0.0860	0.0859	0.0970	0.1050	0.0314	0.0170	0.0665	0.0970
30	0.0241	0.0665	0.0664	0.0757	0.0838	0.0313	0.0290	0.0665	0.0757
31	0.1092	0.0228	0.0657	0.0938	0.0360	0.1225	0.0927	0.0241	0.0938
32	0.0053	0.0971	0.1224	0.1370	0.1171	0.0111	0.0357	0.0971	0.1370
33	0.0055	0.0939	0.1170	0.1302	0.0970	0.0108	0.0360	0.0939	0.1302
34	0.1092	0.0111	0.0850	0.1130	0.0108	0.1225	0.0927	0.0228	0.1130
35	0.0688	0.0834	0.0858	0.0969	0.1029	0.0789	0.0578	0.0664	0.0969
36	0.1046	0.0227	0.0644	0.0908	0.0357	0.1171	0.0906	0.0229	0.0908
37	0.1225	0.0173	0.0936	0.1241	0.0166	0.1371	0.1029	0.0290	0.1241
38	0.0728	0.0837	0.0357	0.0290	0.1029	0.0839	0.0579	0.0688	0.0290

OTUs	28	29	30	31	32	33	34	35	36
28									
29	0.0790								
30	0.0595	0.0241							
31	0.0933	0.0860	0.0665						
32	0.1155	0.0314	0.0313	0.1225					
33	0.1093	0.0295	0.0295	0.1171	0.0108				
34	0.0933	0.0860	0.0665	0.0228	0.1225	0.1171			
35	0.0941	0.0509	0.0509	0.0664	0.0789	0.0757	0.0834		
36	0.0912	0.0828	0.0645	0.0107	0.1171	0.1127	0.0227	0.0644	
37	0.0840	0.0971	0.0758	0.0290	0.1371	0.1304	0.0054	0.0928	0.0285
38	0.0759	0.0533	0.0368	0.0688	0.0839	0.0790	0.0837	0.0533	0.0660

OTUs	37	38
37		
38	0.0939	

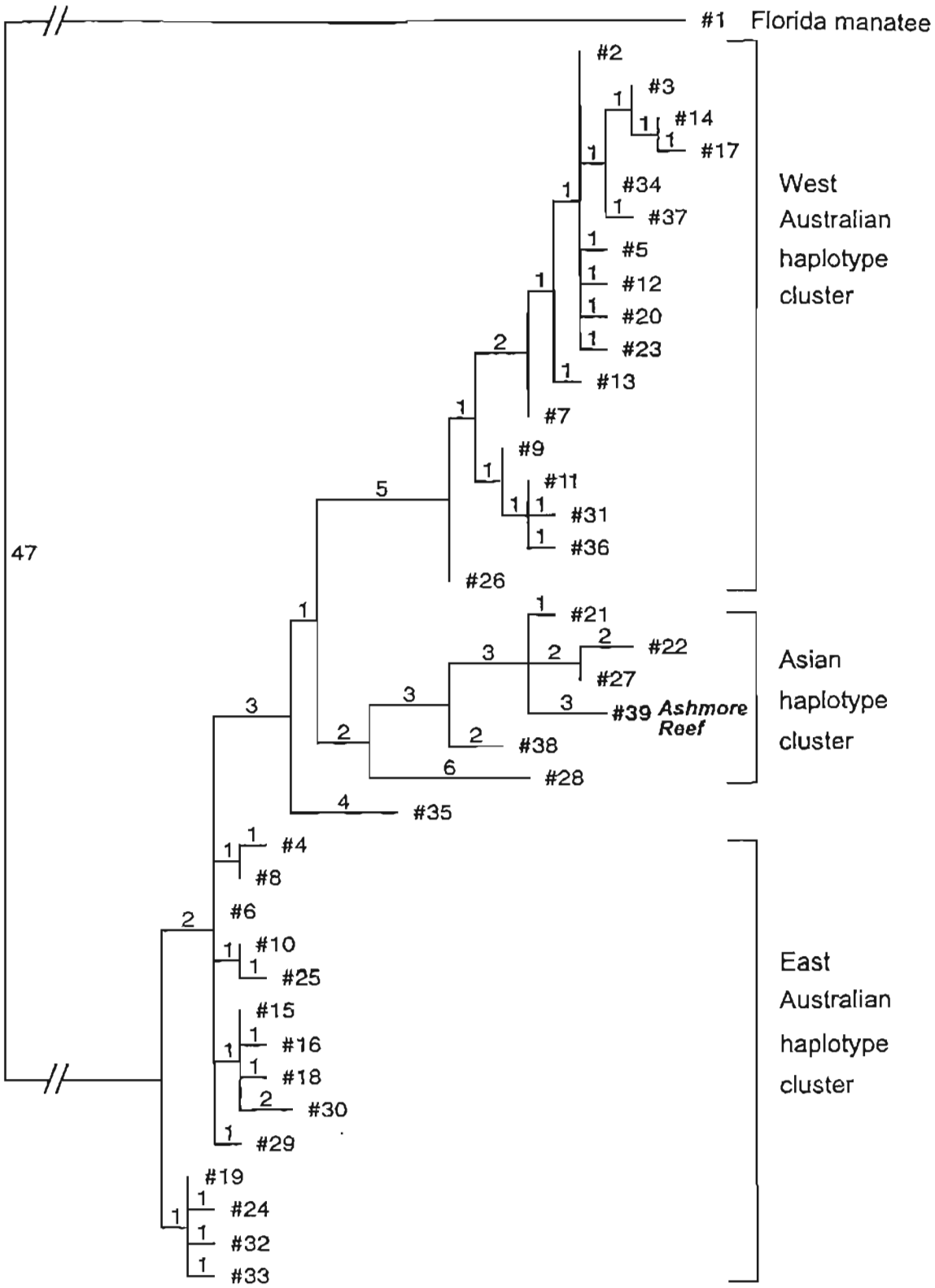
```
                2     8                  24        34 36 39 42    48           60
T.Strait        CCAGTACGGT AGGATTCATG CTCTAAAGCC TAAGTAATTA ATCTCCATTA TACAACCTCT
Ashmore         .T.....A.. .......... .......... ...A.G..C. .C.....C.. .........C
Asia            .T.....A.. .......... ...C...... ...A G..C. .C........ .........C

                61                             90                          113
T.Strait        ACACCATGGA TATTGTCCAG TCCATGTACT TCTTGATCTT GCATAGTACA TTCAACCCTT
Ashmore         G......... .......... .........C .......... .......... ..T.......
Asia            G......... .......... .........C .......... .......... ..T.......

                122                                                  171     179
T.Strait        TATCGTACAT AGCACATCTC TGAGATAGTT CTCGTCAACA CGCTTATCAC CTCCAATGAA
Ashmore         .G......... .......... .......... .......... .......... T.........
Asia            .G......... .......... .......... .......... .......... ........G.

T.Strait        CAGTCCTTGA CTAC
Ashmore         .......... ....
Asia            .......... ....
```

HVR-1 sequence of the Ashmore dugong (unique haplotype). The alignment of the HVR-1 sequence among a Torres Strait (H#2), Ashmore Reef (unique haplotype), and Asian (H#21) dugong. Numbers on the top line indicate nucleotide positions which correspond with the polymorphic sites in table 7.1. A period (.) denotes common sequence, with variable sites to the reference sequence (H#2) indicated by the base concerned. The phylogenetic relationship of the Ashmore Reef dugong sequence is illustrated in a phylogram on the following page.



One of the 172 equally parsimonious (unrooted) mtDNA trees found by PAUP, including the Ashmore Reef dugong (H#39). The same three haplotype clusters are evident as illustrated in figure 7.3: 1) West Australian, 2) East Australian, and 3) Asian. The Ashmore Reef dugong, although a unique haplotype, clusters with the Asian dugongs.