# Population genetics of dugongs around Australia:

## Implications of gene flow and migration



Thesis submitted by
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in the Schools of Tropical Biology, and Tropical Environment Studies and Geography
James Cook University, Townsville.

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# Statement of the contribution of others

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# Glossary of terms

Definitions are adapted from Weaver (2002), Hartwell *et al.* (2000), or Lincoln *et al.* (1987).

**Allele** Alternate forms of a single gene

**Allopatric** Populations, species or taxa occupying different and disjunct geographical areas

**Amino acid** The building blocks of proteins

**Base pair** A pair of bases (A - T or C - G), one in each strand, which occur opposite each other in a double-stranded DNA

**Bootstrap** A statistical method used in molecular phylogenetics based on repetitive sampling with replacement from an original sample to provide a collection of pseudo-replicate samples from which sampling variance can be estimated

**Bottleneck** A sudden decrease in population density with resulting decrease in genetic variability

**Clade** A monophyletic group of taxa sharing a closer common ancestry with one another than with members of any other group of taxa when presented visually as a tree

**Cloning** The process of making copies of a specific piece of DNA

**Competent cells** The state of bacterial cells able to take up DNA from their medium

**DNA** Deoxyribonucleic acid, the molecule of heredity that encodes genetic information

**DNA sequencing** The determination of the exact order of the base pairs in a segment of DNA

**Evolutionary Significant Unit (ESU)** Populations that have adapted to local conditions and indicates a reservoir of unique genetic variability, demonstrated by divergence at both mtDNA and nDNA (Moritz, 1994a)

**Gel electrophoresis** A process by which molecules are separated by electrical current according to size and electrical charge on a gel, usually solid matrix of agarose or acrylamide

**Gene** The functional and physical unit of heredity passed from parent to offspring, usually corresponding to a protein or RNA product

**Genetic drift** The occurrence of random changes of gene frequencies not due to selection, mutation or immigration

**Genetic marker** A segment of DNA with an identifiable physical location on a chromosome, the inheritance of which can be traced

**Genome** The entire collection of genetic information in each cell of an organism

**Genotype** The actual alleles present in an individual at a locus or across many loci

| | |
|---|---|
| **Haplotype** | A unique DNA sequence usually referring to mtDNA |
| **Heteroplasmy** | More than one genetic type in an individual |
| **Heterozygous** | A genotype in which the two copies of a gene that determine a particular trait are different |
| **Homolog** | Genes that have evolved from a common ancestral locus |
| **Homozygous** | A genotype in which the two copies of a gene that determine a particular trait are the same |
| **H (heavy)-strand** | The outer strand of the mtDNA genome (double stranded) |
| **Intron** | The non-coding regions of a gene that is transcribed into RNA but excised during the processing of the primary RNA into mature mRNA |
| **L (light)-strand** | The inner circular strand of the (double stranded) mitochondrial DNA genome |
| **Management unit** | Populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of alleles (Moritz, 1994a) |
| **Microsatellite** | A DNA element composed of 15 - 100 tandem repeats of two to six bp motifs |
| **mRNA** | Messenger RNA transcript which comprises the genetic information for making one or more proteins when translated |
| **Mutation** | heritable alterations of the genetic material occurring in gamete-producing cells |
| **Negative control** | PCR reagent sample to which no DNA has been added, used to indicate contamination of PCR reagents |
| **Nucleotide** | A subunit of DNA or RNA consisting of a nitrogenous base, a phosphate and a sugar molecule |
| **Oligonucleotide** | **S**hort, synthesised chains of nucleotides used as DNA probes and primers |
| **Panmictic** | Pertaining to an infinitely large randomly interbreeding population |
| **Phenotype** | The observable traits or characteristics of an organism |
| **Phylogeny** | The evolutionary history of lineages or species |
| **Polymerase chain reaction (PCR)** | Amplification of a region of DNA using flanking primers by repeated cycles of DNA polymerase actions |
| **Polymorphism** | Any variation in the sequence of DNA among individuals |
| **Population** | For definitions see section 2.1.1. |
| **Primer** | A short oligonucleotide sequence used in the polymerase chain reaction to initiate the synthesis of DNA by a DNA polymerase |
| **Recombination** | The exchange of gene segments between non-sister chromatids through the physical exchange of homologous strands of DNA |

**Restriction enzymes** Enzymes that recognize a specific sequence of double-stranded DNA and cut the DNA at that site

**Restriction fragment length polymorphism (RFLP)** A variation between individuals in the number of cutting sites for a given restriction endonuclease in a given genetic locus

**Sequencing** Determination of the amino acid sequence of a protein or the base sequence or DNA or RNA

***Taq* Polymerase** A thermostable DNA polymerase obtained from the thermophilic bacterium *Thermus aquaticus*. Used for amplification of DNA via the polymerase chain reaction

**Universal primer** An oligonucleotide designed to be complementary to target sequences that are conserved over a wide range of taxa

# Abstract

Dugongs (*Dugong dugon*) live in tropical inshore areas around Northern Australia and throughout the Indo-West Pacific. These marine mammals are obligate seagrass feeders and are thus largely restricted in distribution to areas of seagrass habitat. The turbid environment and the lack of a dorsal fin for individual identification makes it impractical to study these animals using standard observational methods. Consequently very little is known about dugong population structure and migration patterns. From satellite tracking of individuals and aerial surveys it appears that dugongs, like many other marine mammals, can move long distances, but the timing and length of movements vary individually. If dugongs move and mate in their new locality, there will be substantial gene flow across large spatial scales and very little population differentiation around the tropical Australian coast. However, if dugongs maintain a home range and are philopatric (*i.e.* any long distance movements are for food or other reasons not including mating), then there would be very limited gene flow and substantial structuring among populations. In order to ascertain which pattern of population structure is present in the dugong, I used a variety of molecular techniques to assess dugong population genetic structure around the North Australian coast.

A comparison of the two published mitochondrial genomes of dugongs showed that most variation was contained within the control region. I subsequently characterised the entire control region of eight individual dugongs (four available from previous studies and four sequenced in this study) and identified the amount of variation among them. I also made a comparison of the mitochondrial control region of dugongs with available sequences of their closest relatives, the paenungulates (Proboscidea, Hyracoidea, and other members of the Sirenia), in order to assess whether the control region was capable of producing reasonable phylogenies and its usefulness as a marker for phylogeographic and population studies on the dugong.

The 5' domain of the control region was identified as the most appropriate section of this locus for use in the phylogeographic analysis. This domain demonstrates high diversity in the dugong. Phylogeographic analysis of the 492 bp alignment of 115 dugongs, identified two divergent Australian mitochondrial lineages. I hypothesise that the Australian lineages diverged historically during periods of low sea level that would have reduced habitat availability and produced geological barriers such as the Torres

Strait land bridge between Northern Australia and Papua New Guinea. One lineage is restricted geographically to the coast of Queensland and into the Northern Territory, while the other is more widespread occurring from Shark Bay in Western Australia to Moreton Bay in Queensland (*i.e.* across the entire Australian range). The widespread lineage is poorly represented in Southeastern Queensland. Given the availability of continuous habitat with higher sea level for the past 7000 years and the high mobility of dugongs, I expected that more complete geographic mixing of lineages would have occurred. Mitochondrial DNA sequences of dugongs from Asia are distinct from those of Australian dugongs. These results suggest long-term isolation between dugong lineages and subsequent partial geographic mixing of dugong matrilines.

Nuclear DNA microsatellite loci isolated from the Florida manatee were tested for use as population genetic markers the dugong. These loci displayed considerable allelic diversity in the dugong, significantly greater than observed in the Florida manatee. For example, 27 alleles were identified in the dugong at locus TmaA04, while only one allele was identified in the Florida manatee at the same locus. These microsatellite markers reveal a high level of gene flow among dugongs in Australia and a significant level of isolation-by-distance across Australia.

Comparison of the results from mtDNA and nDNA indicate that members of the two distinct mitochondrial lineages within Australia interbreed in areas where they overlap geographically. The contrasting patterns of structure presented by the mtDNA and nDNA suggest male-biased gene flow in the dugong. This pattern has not yet been identified from ecological studies, but is consistent with common dispersal patterns in mammals.

The major findings of this study are the detection of 'healthy' levels of genetic diversity of Australian dugongs, a suggestion of male-biased gene flow, and a demonstration of significant gene flow around Australia. This high level of gene flow makes the allocation of management units difficult. These results indicate the importance of a co-ordinated management strategy at a spatial scale of thousands of kilometres. Dugongs within Australia cannot be managed at a bay level due to the connectivity between bays observed in this study. Co-operation between management agencies at local, state, national and international spatial scales is required in order to conserve this vulnerable species.

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# Chapter 1: General introduction

## 1.1 The role of genetics in conservation biology

Conservation biology is concerned with the application of science to address the biology of species, communities and ecosystems affected (either directly or indirectly) by human activities (Soulé, 1985). The main objective of conservation biology is to provide principles and tools for preserving biological diversity (Soulé, 1985). Conservation biology spans disciplines including, but not limited to: social science, ecophysiology, environmental monitoring, historical biogeography, population biology and genetics (Soulé, 1985). Frankham *et al.* (2002) lists 11 major genetic issues in conservation biology. The present study investigates three of these aspects in relation to the vulnerable marine mammal, *Dugong dugon*, around Australia: fragmentation of populations and loss of gene flow, definition of management units within species, and the use of genetics to gain understanding of aspects of species biology important to conservation.

## 1.2 Introduction to Sirenia

The dugong is unique among mammals in that it is the only fully marine herbivore and the only extant member of the Dugongidae. The Dugongidae is one of two families in the Order Sirenia, the other being the Trichechidae (the manatees). The Trichechidae contains the West Indian manatee (*Trichechus manatus*), the Amazonian manatee (*T. inunguis*) and the West African manatee (*T. senegalensis*). The West Indian manatee is divided into two subspecies, the Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*). All members of the Trichechidae require fresh water to survive, with the Amazonian manatee being an obligate freshwater species, while the dugong is exclusively marine (Martin and Reeves, 2002). Steller's sea cow, the only other dugongid of recent times, was discovered and hunted to extinction within 27 years during the eighteenth century (Marsh *et al.*, 2002). It is suggested that the dugong is the most likely to survive out of all the sirenians, as manatees have a much more restricted range, and lower population numbers than those recorded for the dugong in Australia (Marsh *et al.*, 2002). However, all extant species in the Sirenia are regarded as

vulnerable to extinction (Hilton-Taylor, 2000) and are listed on Appendix I of the *Convention on International Trade of Endangered Species* (CITES).

The dugong has a wide geographical range that spans some 47 countries and territories (Figure 1.1) (Marsh *et al.*, 2002; H. Marsh pers. com. 2005). It is now represented by relict populations throughout the Indo-West Pacific which are separated by large distances and are reported to be declining (Marsh *et al.*, 2002). The need for information on dugong genetic diversity to aid in multi-jurisdictional management is obvious given the current state of knowledge of dugong biology, movement patterns and threatening processes.

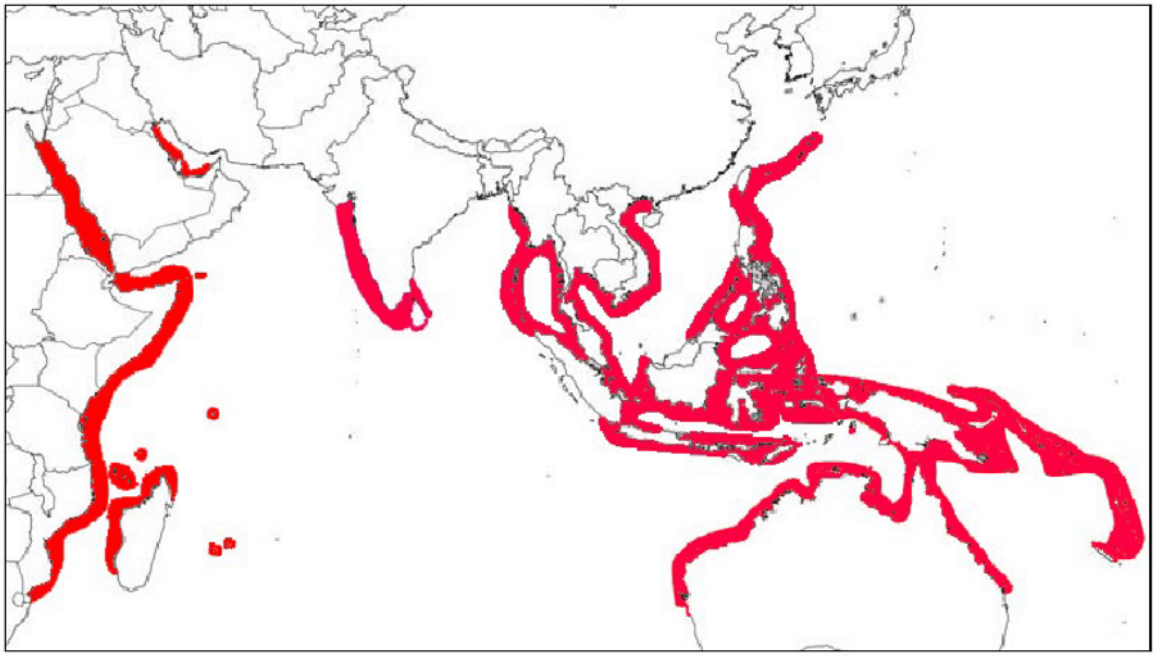


**Figure 1.1** Map showing the distribution of the dugong as shaded regions around the coastline. Taken from Marsh *et al.* (2002).

## 1.3 Threatening processes and management initiatives in place

Dugongs are vulnerable to anthropogenic impacts due to their long life span (approximately 70 years) and breeding cycle (13 months gestation, 18 months suckling and 3 - 7 years between calving) (Marsh, 1995). Population simulations conducted by

Marsh (1986) indicate that even under optimal conditions populations of dugongs are unlikely to increase in size more than 5% per year. Along Queensland's east coast there has been a significant decline in numbers (Marsh *et al.*, 1996; Marsh *et al.*, 2005). It is this decline that is of concern to the general public, environmental managers and scientists alike. At present, it is not known whether the populations in Western Australia and the Northern Territory are also declining, as these areas are data deficient.

### *1.3.1 Indigenous hunting*

Traditional Indigenous hunting has occurred throughout the range of dugongs, but since 1997 has been prohibited along the Queensland coast south of Cooktown (Marsh, 2000). Very little information on the catch rates of dugongs around Australia exists due to the difficulty of obtaining this information. However the catches of a limited number of the Indigenous communities that partake in dugong hunting in Torres Strait have been monitored by an Australian government agency intermittently since 1991 (Marsh *et al.*, 1997). Recent analyses on the catch rates in the context of estimates of the population size and demography have indicated that the dugong fishery in Torres Strait is currently unsustainable (Heinsohn *et al.*, 2004; Marsh *et al.*, 2004). This current scientific evidence has prompted the Australian government to undertake a strategic assessment of the risks to dugongs of traditional hunting in Torres Strait.

### *1.3.2 Loss of seagrass habitat*

One of the greatest threats to dugongs throughout their range is the loss of their major food source and habitat due to both natural and anthropogenic causes. Dugongs feed almost exclusively on seagrass by uprooting whole plants or feeding on the leaves when plants can not be uprooted (Anderson, 1982a). As seagrass specialists the distribution of dugongs is largely confined to inshore seagrass habitats. Seagrass is sensitive to human influences (Poiner and Peterken, 1996) and can be destroyed directly by mining, trawling, dredging, and boat propeller scarring (Kirkman, 1997). Seagrass meadows can also be disturbed as a result of inland and coastal clearing, and land reclamation, all of which cause an increase in sedimentation and turbidity resulting in a lower level of light available to the seagrass plants (Kirkman, 1997). Sewage, detergents, pesticides, herbicides, fertilisers and heavy metals are also threats to the inshore seagrass habitat

(Haynes *et al.*, 2000a; Haynes *et al.*, 2000b). Natural disturbances such as cyclones and floods can also result in episodic losses of seagrass (Poiner and Peterken, 1996), which can have a large effect on the local dugong population (Preen, 1993; Preen and Marsh, 1995).

The only management initiatives in Australia that have been widely implemented to protect seagrass habitat is through the creating of marine parks, and fishing industry closures to prevent structural damage to seagrass meadows (Lee Long *et al.*, 2000; Marsh *et al.*, 2002). Additional measures to protect seagrass have been implemented along the Queensland coast. These include the establishment of Dugong protection areas, the Representative Areas Program, Reef Water Quality Protection Plan and an upgrade of the sewage treatment plans. Dugong protection areas have been established in seagrass areas used by dugongs within the Great Barrier Reef Marine Park (GBRMP) and Hervey Bay, controlling the types of fishing gear that is able to be used (Marsh, 2000), providing limited protection to seagrass meadows. Additionally, the increase of no-take areas within the GBRMP to 33% under the Representative Areas Program increases the protection level provided to seagrass from fishing impacts (http://www.reefed.edu.au/rap/). The threat to seagrass meadows from terrestrial sources is being addressed by the Commonwealth and Queensland Governments, who have a memorandum of understanding to develop and implement a Reef Water Quality Protection Plan (http://deh.gov.au/coasts/pollution/reef/index.html). Standards for the treatment of sewage on island resorts and sewage outfalls within the Great Barrier Reef World Heritage Area (GBRWHA) has also been introduced, along with upgrades to the treatment of sewage in Moreton Bay (Marsh *et al.*, 2005).

### *1.3.3 Fisheries*

Another, largely unquantified, threat to dugongs is that they may become entangled in fishing nets and traps (Perrin *et al.*, 1996). Shark nets along the Queensland coast, introduced as a measure to protect bathers from sharks, have also been identified as a problem. Through hind casting Marsh *et al.* (2005) have found that the bycatch takes in shark nets has decreased by approximately 97% since the 1960s when shark nets were introduced. This study by Marsh *et al.* (2005) highlighted the problem of nets, including shark nets. Although this analysis relies on numerous explicit assumptions, it is clear

that the number of dugongs being captured in shark nets has declined over the years although the extent of this decline is not known. To avoid by-catch in shark nets but still maintain bather protection, the nets have been replaced with baited hooks at a number of locations along the Queensland coast. The use of pingers (acoustic alarms) on fishing nets is also being tested to determine if these would help dugongs avoid nets (Marsh *et al.*, 2002). In 1997, the Commonwealth and Queensland Governments established 16 Dugong Protection Areas (DPA) within the GBRWHA and Hervey Bay (Marsh, 2000) These areas were designed to protect dugongs from direct anthropogenic effects such as drowning in fish nets and boat strikes (Marsh, 2000). Current restrictions on fishing within the GBRMP under the Representative Areas Program protects 80% of dugong seagrass habitat from trawling (Grech and Marsh, in review).

### *1.3.4 Other threats*

There are numerous other threats encountered by dugongs including vessel strikes, ecotourism, disease and chemical pollutants (Marsh *et al.*, 2002). However adequate study into the effects of these activities on the dugong has not been carried out.

## 1.4 Can genetics contribute to dugong conservation?

The elusive behaviour and long life span of the dugong along with the mostly turbid tropical waters and isolated areas they inhabit, make obtaining vital information on movement and mating strategies of dugongs difficult using traditional techniques. Satellite tracking of individuals has shown that dugongs are capable of moving large distances (Sheppard *et al.*, in press). Sheppard *et al.* (in press) identified that 44 dugongs of 72 that have been fitted with satellite tags made long distance movements of up to 560 km. However, these satellite-tagging studies occur over a short time period relative to the lifespan of an individual. The limited data these studies provide are hard won and require considerable time and expense to obtain. Genetic techniques can provide insight into directly measurable parameters such as the sex of an individual, or inferred parameters like the population and herd structure, and movement of individuals or at least their genes.

A greater understanding of the long-term movement patterns between geographic locations can be gained from patterns of gene flow. Non-invasive or minimally invasive sampling means that such information can be gained with little stress to the animals. Genetic fingerprinting studies allow the identification of individuals, populations and can assign parentage. Broader scale studies allow estimates of historical and present effective population sizes. This information, in conjunction with current ecological studies, will inform management activities, and further enhance the chances of dugong survival.

### *1.4.1 Previous molecular genetic studies on dugongs*

There are only three published studies to date on genetic variability of members of Sirenia. All were conducted on manatees, two on the West Indian manatee (*Trichechus manatus*) (McClenaghan and O'Shea, 1988; Garcia-Rodriguez *et al.*, 1998) and one on the Amazonian manatee (*T. inunguis*) (Cantanhede *et al.*, 2005). These studies are discussed in chapter 2, section 2.10. A preliminary study of dugong phylogeography is the only work previously conducted on dugongs (Tikel, 1997). From her initial data, Tikel (1997) suggested the presence of two mitochondrial lineages among Australian dugongs. This study, however, was limited in sample size from a number of important locations and also in the length of DNA fragment that was analysed (approx 194 bp).

## 1.5 Outline and aims of thesis

### *1.5.1 Aims of thesis*

In this project, I aim to assess the degree of genetic structuring among dugong populations in the waters of Northern Australia. By identifying population genetic structure, important ecological information such as the extent to which interbreeding occurs between adjacent geographical localities can be estimated. This information will not only complement our understanding of the ecology of dugongs, but will also contribute significantly to the development of management strategies that will enhance the conservation efforts aimed at the ongoing survival of this species.

### *1.5.2 Outline of Thesis*

This thesis consists of seven chapters visually depicted as a flow diagram (Figure 1.2). The first two chapters provide background information. This chapter places the study in a conservation context by outlining the status of the dugong, and threats that this species faces. The overall aim of the thesis, and thesis overview are also provided in this chapter. Chapter two provides an introduction to the usefulness, theories and potential problems associated with molecular genetic studies on marine mammals.

Chapter three describes the characterisation of the mitochondrial control region and assesses the potential of using this marker in a phylogeographic and population study of dugongs. This chapter also describes the genetic differentiation of the control region between dugongs and their closest relatives, members of the superorder Paenungulata. These analyses place the variation of the mitochondrial control region among dugongs into an evolutionary context.

Results of the phylogeographic study on dugongs, with an emphasis on populations around Australia, are described in detail in chapter four. This study has provided clear evidence of the existence of two maternal lineages of dugongs around Australia, which overlap in distribution in the GBRWHA and Torres Strait. Possible explanations of the identified pattern are also discussed. Some demographic parameters are also inferred from the phylogeographic data.

Six microsatellite markers developed for the Florida manatee were used in a detailed study of the population structure of dugongs around Australia, which is described in chapter five. No geographically structured populations were identified using microsatellite loci. However, isolation-by-distance was apparent around Australia. This chapter also inferred that gene flow occurs between dugongs in Australia and other countries.

In chapter six, a comparison of the results from the mitochondrial DNA and microsatellite loci is presented. This comparison showed that members of the distinct

maternal lineages in Australia are interbreeding in the areas where they overlap geographically. Similarly, the different patterns associated with the two genetic markers indicate male-biased gene flow in the dugong.

The implications of these results for management are then discussed in chapter seven. The management initiatives already in place around Australia are assessed in light of the new information.

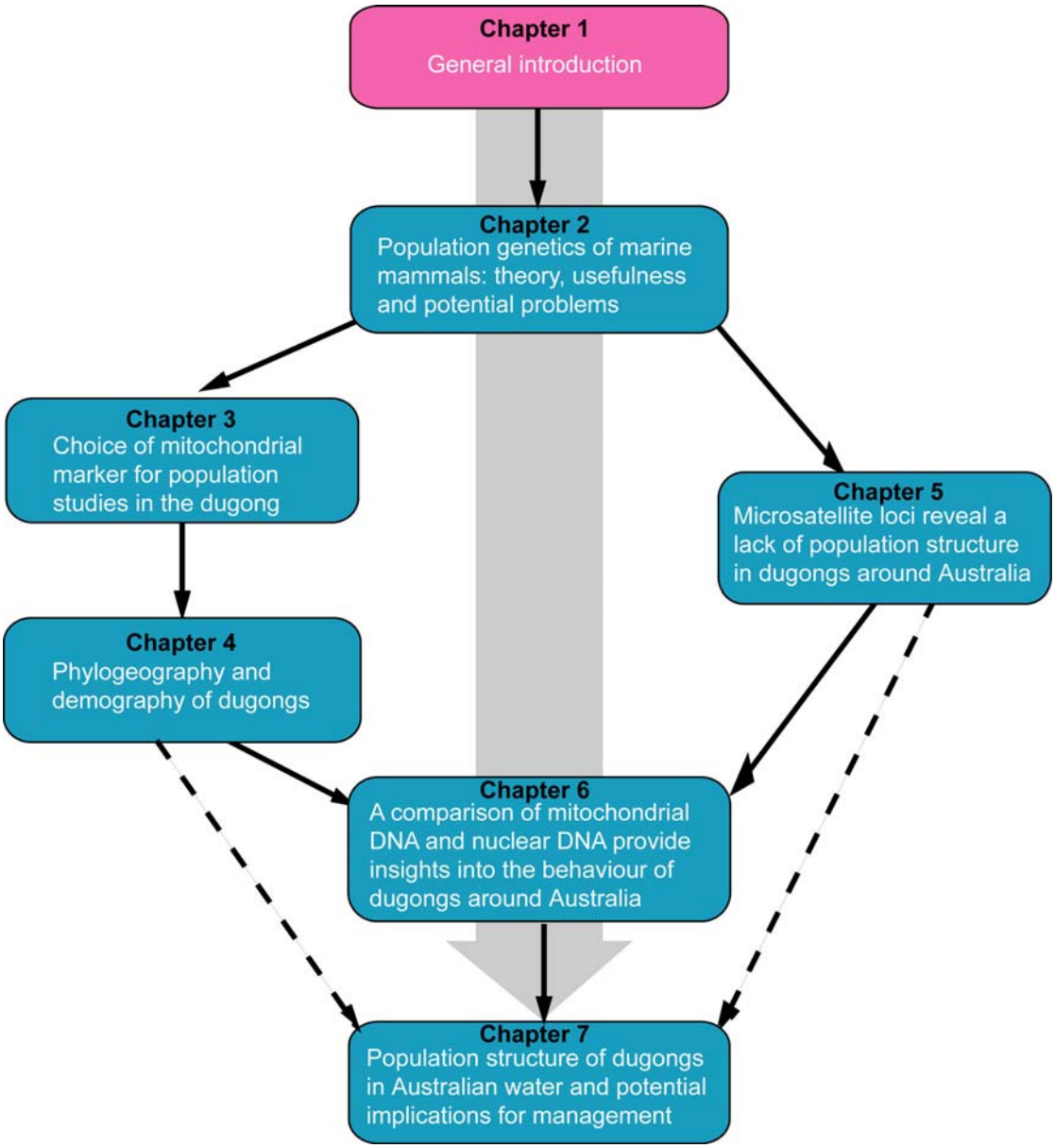
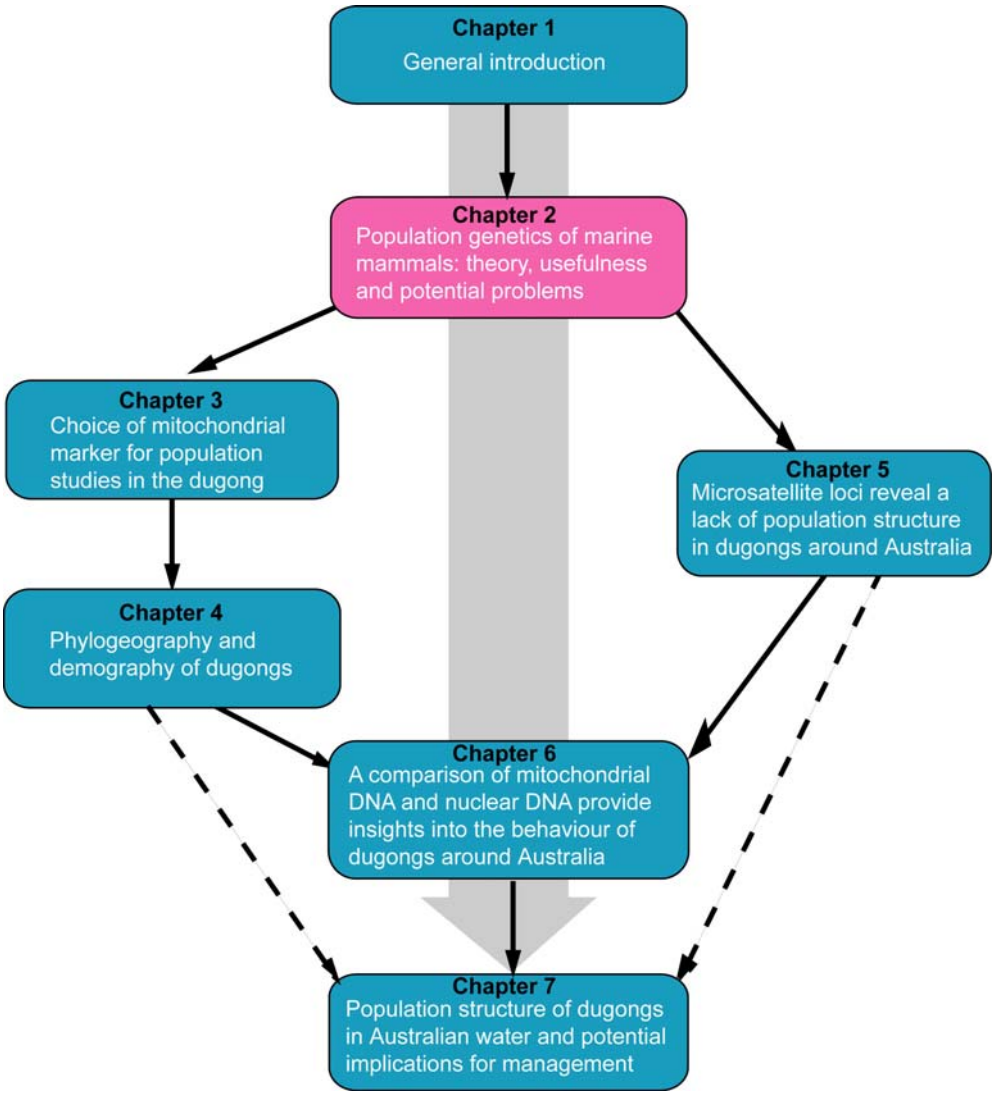



**Figure 1.2** Flow-diagram showing the inter-relatedness of each chapter of this thesis. The current chapter is highlighted. This diagram will be placed at the start of every chapter with the relevant chapter being highlighted.

# Chapter 2: Population genetics and phylogeography of marine mammals: theory, usefulness and potential problems

*All marine mammals have relatively similar life history characteristics. They are relatively large, long lived animals, and invest a lot of time and energy into the care of their young. They also have long generation times and a slow intrinsic rate of population growth. Additionally, marine mammals can potentially travel large geographic distances quickly. This chapter outlines the utility, theories and potential problems associated with genetic studies on marine mammals.*



## 2.1 What is a population?

The traditional ecological definition of a population is "*a group of individuals of one species in an area*" (Begon *et al.*, 1986). A genetic population can be defined as a group of conspecific organisms that share greater kinship with each other than with the members of other similar groups (Hoelzel and Dover, 1991). In an ecological study different populations are invariably allopatric. However, in genetic studies two populations may be sympatric but still distinct. An example of this is a study by Hoelzel (1991) who identified two genetically distinct sympatric communities of killer whales (*Orcinus orca*) in the waters of British Columbia, Canada, using mitochondrial DNA sequence data and RFLPs. Three communities were identified by ecological studies previous to Hoelzel's study: two resident and one transient. The two resident communities were found to be one distinct unit that differed genetically to the transient community (Hoelzel, 1991). The degree of genetic differentiation between the resident and transient killer whales of British Columbia is similar to the differentiation found between killer whales in the Pacific and Atlantic Oceans (Hoelzel, 1991).

One key factor that needs to be identified when defining a genetic population is the capability of an individual to move (vagility). The level of vagility between groups of individuals can have an impact on the genetic differentiation of the groups. Theoretically only one individual needs to move between groups every generation to produce an interbreeding population and homogenise any genetic structure in a population/species (Lowe *et al.*, 2004). For marine mammals, which are highly mobile, one would expect limited genetic differentiation between groups, as movement between groups is not restricted. However, this is not the case for numerous species of marine mammals (see section 2.6 and 2.7). One example of this is the humpback whale (*Megaptera novaeangliae*), which covers large distances (average 10 000 km per round trip) between feeding and breeding grounds annually, but still exhibits strong population structure between stocks (Baker and Palumbi, 1997).

### *2.1.1 Definitions of population used in this thesis*

Many genetic studies are motivated by an interest in the identification of distinct groups with an aim to identify 'populations'. In such studies an ecological definition of a

population may be used initially until the genetic kinship of individuals is identified. I will initially define dugong populations using an ecological definition, with individuals found in a geographical location being a 'population'. A geographical location is an area, namely a town or distinct bay, closest to where the dugong was found. Genetically distinct groups of dugongs will be referred to as a 'genetic population'. Because of the opportunistic way I was obliged to obtain samples (see section 2.8), several different groupings of samples were used for various analyses. In this study, groups of samples from neighbouring 'populations' are referred to as 'demes'. The term 'deme' in this thesis are defined using the ecological definition of a local grouping of individuals from a species. 'Regions' are defined as the grouping of neighbouring 'demes'. The term 'metapopulation' is defined and used to mean a number of geographically isolated subpopulations which are connected by limited gene flow (Begon *et al.*, 1986; Gliddon and Goudet, 1994). The assignment of different groupings, results from the limited information available on dugong migration, movement patterns and population boundaries, and will be refined during the course of the work described here.

## 2.2 How can we study populations?

Many studies of natural animal populations have traditionally used variations of two methods: mark-release-recapture (tagging studies) and observational methods. Both these approaches are time consuming in comparison with molecular methods such as those used in this thesis.

### *2.2.1 Mark-release-recapture and tagging studies*

The long life span, large size, vagility and geographic distribution of marine mammals makes tagging and mark-release-recapture studies difficult. Populations of marine mammals are spread throughout the oceans, and most are known to go on long distance migrations to feeding and breeding areas annually (Marsh *et al.*, 1993). This makes the spatial scale of population studies on marine mammals very large.

Tags, suitable for marine mammals that can be identified from a boat a good distance away are difficult to find. Anderson (1982b) used a paint stick to place temporary tags on dugongs in Shark Bay. These were useful for a short time. However the use of such

visual tags is limited in turbid water such as that found on the east coast of Australia (Marsh and Rathburn, 1990). It has been shown that plastic cattle ear tags, turtle tags, and passive integrated transponder tags (PIT) can be used for mark-recapture programs of dugongs (Lanyon *et al.*, 2002). However, the study Lanyon *et al.* (2002) is being carried out in an area with clear water and large herds of dugongs, permitting relatively easy sighting and physical capture of animals. The practicality of such an approach in turbid water is still to be evaluated.

The ability to attach satellite transmitters to marine animals has greatly enhanced the information that ecologists and biologists have gained. These tags have provided information on the everyday behaviour of individuals. However, the expense (and in some cases constraints regarding permits) results in only a small number of individuals being tagged in any single study. An example of the limited sample size is a study by de Iongh *et al.* (1998) who tagged four dugongs near Haruku Island in East Indonesia. These dugongs were tracked from 41 days to 285 days, and showed individualistic patterns of movement, travelling between seagrass meadows up to 65 km apart. The usefulness of the data obtained is severely limited by sample size. Therefore, making appropriate interpretations about the entire population from these studies is difficult.

### *2.2.2 Observational studies*

Numerous observational studies have been conducted on marine mammal populations. These include studies utilising photo-identification, and behavioural information. Photo-identification studies rely on the photographer's ability to produce photos that allow distinct identification of individuals. Distinguishing marks on an individual may not always be present. Marten and Psarakos (1999) took 1 800 underwater photographs of wild spinner dolphins (*Stenella longirostris*) and established identification of only 125 individuals. There is always the possibility of identifying marks on fins disappearing if a shark attacks an individual, or the gain of additional scars and marking during an individual's lifetime. Photo-identification of dugong individuals has not been possible to date due to the lack of a dorsal fin, the lack of aerial display behaviour, and the turbid waters they inhabit throughout most of their range.

Photo-identification studies can be conducted in conjunction with behavioural studies providing substantial information from one 'population' of the species in an area. One such study was conducted by Reid *et al.* (1991) who catalogued 891 individually identifiable manatees in Florida based on the number and pattern of scars, colour and extent of tail mutilations. The catalogue was updated annually due to the change in scarring patterns as a result of slow healing or the further accumulation of scars (Reid *et al.*, 1991). A total of 53% of manatees were resighted, which allowed for a greater understanding of the manatees' seasonal migrations between study areas (Reid *et al.*, 1991). Photo-identification of Florida manatees was also used to estimate their survivorship (Langtimm *et al.*, 2004) and their reproductive rate (Kendall *et al.*, 2004).

### *2.2.3 Population genetic studies and the problems with traditional methods of studying populations*

One major problem with these traditional methods of studying populations is the amount of time required to gain information - typically at least several years. Furthermore, the traditional methods provide only a small amount of information relative to that gained from molecular studies (Avise, 1994). An example is a study reported by Bigg *et al.* (1990), who followed two sympatric forms of killer whales for 15 - 20 years. Bigg *et al.* (1990) identified two distinct eco-types based on their diet, the residents and the transients. A relatively large genetic distinction between the eco-types was identified later as indicated in section 2.1. Population genetic methods also allow the study to be conducted over several timeframes depending on the markers used and type of analysis conducted (Avise, 1994), from evolutionary scales to present day population processes. By studying the genetics of a population a great deal of information can be gained relatively quickly in comparison to more traditional methods.

## 2.3 What is population genetics?

Population geneticists are concerned with the genetic basis of evolution (Gillespie, 1998). Fundamentally, evolution is the result of progressive change in the genetic composition among individuals in a population, or a number of populations (*e.g.* Hartl and Clark, 1989; Freeman and Herron, 2004). The interaction between a study organism's biology and its environment initiates a progressive change in genetic

composition. In order for population geneticists to get a complete picture they are required to utilise available information on the number of individuals, mating patterns, geographic distribution, migration and natural selection of the study organism (Hartl and Clark, 1989). Population genetic studies characterise natural populations and try to explain their evolution and to predict the impact of various natural and anthropogenic processes on the genetic composition of the population. Hence, the ultimate goal of population genetics is to identify the genetic variation that exists in natural populations and to explain this variation in terms of its origin, maintenance, and evolutionary importance (*e.g.* Hartl and Clark, 1989; Freeman and Herron, 2004). Population genetics can be applied to organisms in a variety of ways, including studies on genetic diversity, population genetic structure, mating systems, parentage, stock structure, phylogeography and gene flow. This study is concerned with identifying the genetic diversity, phylogeography, population genetic structure, and gene flow within dugongs around Australia.

In order to fully understand population genetics, the basic theoretical principles need to be understood and are outlined in section 2.4. Some fundamental tools of population genetics are mathematical models based on these principles. These models are used as a comparison to natural populations in order to gain an understanding about the processes responsible for the genetic patterns identified (Gillespie, 1998).

## 2.4 Theory behind population genetics

### *2.4.1 Hardy-Weinberg equilibrium (HWE)*

In large random mating populations, the allele frequencies at an autosomal locus not under selection will attain equilibrium. This equilibrium is referred to as the Hardy-Weinberg equilibrium after Hardy (1908) and Weinberg (1908) who first discovered it. This simple concept is an underlying principle of population genetics and is commonly used as an assumption for analyses. It is implemented as a comparison of allele frequencies in the population for each locus compared to the expected frequencies under HWE model and is tested by chi-squared ($\chi^2$) analysis. Deviations from the expected allele proportions under HWE usually imply that one or more population structuring

processes are operating such as non-random mating, migration or selection (Frankham *et al.*, 2002).

The Hardy-Weinberg principle makes the following assumptions about the population: 1) the organism in question is diploid, 2) reproduction is sexual, 3) generations are non-overlapping, 4) mating is random, 5) the population size is very large, 6) migration is negligible, 7) mutation can be ignored, 8) natural selection does not affect the locus under consideration.

The usefulness of the Hardy-Weinberg equilibrium is limited because of the low probability of natural populations fitting all of the assumptions. Marine mammals may not fit the Hardy-Weinberg assumptions for a number of reasons. As marine mammals have long generation times it is likely those generations overlap, therefore violating assumption number three of the Hardy-Weinberg equilibrium. Only fast-growing short-lived organisms such as insects and some annual plants are capable of meeting this assumption. For some marine mammals, such as the vaquita (*Phocoena sinus*) (Taylor and Rojas-Bracho, 1999), population sizes are small, thus the effects of migration, drift and natural selection will be significant. The fact that many whales undergo seasonal migration is another reason why some marine mammal populations may not fit the assumptions of Hardy-Weinberg equilibrium. In this case assumption six is violated.

For dugongs, it is anticipated that a number of the assumptions of Hardy-Weinberg equilibrium will not be met, especially the assumptions of non-overlapping generations and random mating. The generation time of dugongs is estimated to be approximately 27 years (H. Marsh pers. com. 2005); therefore the generations overlap in the population, not meeting assumption three. Dugongs on the east coast of Australia have a promiscuous mating system with the female being surrounded by a number of males at mating time (Preen, 1989). In theory this mating system should be a form of random mating. However on the west coast of Australia the dugong males defend territories called ‘leks’ in the hope of attracting a female (Anderson, 1997). Dugongs on the west coast of Australia are unlikely to mate randomly, therefore not meeting assumption

number four. For further discussion on the Hardy-Weinberg equilibrium assumptions in dugongs see section 5.4.2.

### *2.4.2 Effective population size ($N_e$)*

The effective size of a population is the average number of individuals in a population which are assumed to contribute genes equally to the succeeding generation (Lincoln *et al.*, 1987). The importance of this concept lies in its utility for predicting the dynamics of genetic variation within and among populations (Chesser *et al.*, 1993). The effective population size gives a measure of the rate of genetic drift and inbreeding within a group of individuals (Caballero, 1994). It can also be used to monitor genetic variation in natural populations and allows the prediction of the impact of management practices on genetic variation (Caballero, 1994).

There are three measures of effective population sizes: the inbreeding effective size, the variance effective size and the eigenvalue effective size. The basis for calculating each of these values differs. The inbreeding effective size is based on the probability of homozygosity because of common ancestry, the variance effective size is based on changes in the variance of allele frequency drift per generation, and the eigenvalue effective size is based on the asymptotic rate of decay of segregating loci (Crow and Denniston, 1988). The most commonly used measure of effective population size is the inbreeding effective size. This measure is calculated using the inbreeding coefficient, which is the probability that two gametes, which unite to produce a zygote, carry identical-by-descent copies of a gene (Wright, 1922).

The assumptions surrounding calculations of effective population size include random mating in the population, the population is subdivided into infinite number of subpopulations with each subpopulation having a constant number of breeding individuals per generation, an infinite number of male and female gametes, non-overlapping generations, and that all individuals survive from birth to adulthood (Wright, 1931). Marine mammals are unlikely to meet the assumptions inherent in the calculation of effective population size. The long life and social structure of marine mammals prevent non-overlapping generations or random mating as discussed above

(section 2.4.1). In any natural population it is also unlikely that all individuals of a generation survive to adulthood. Accordingly there have been modifications to the calculations of effective population size to allow for deviations of the assumptions listed above depending on the biology and ecology of the organism in question [reviewed in Caballero (1994)].

Slade *et al.* (1998) calculated the total effective population size ($N_e$) and the female effective population size ($N_f$) for southern elephant seals (*Mirounga leonina*). From these estimates they identified the effective to current census population ratio of the southern elephant seal to be 0.05, which is reflective of its highly polygynous breeding system. The effective population size for the southern elephant seal is lower than would be expected from observational data as estimates of copulatory success and lifetime reproductive success are over estimated (Slade *et al.*, 1998). This study stresses the usefulness of a genetic insight into a species' biology that may aid management authorities.

### *2.4.3 Genetic diversity*

Genetic diversity is variation, or polymorphism, in any inherited trait within a taxonomic group. Such variation usually corresponds to differences in DNA sequences. An understanding of the levels of genetic diversity within a population will allow for an understanding of the genetic 'health' of the population. Higher levels of genetic diversity may allow populations to adapt to environmental changes more efficiently than populations with little or no genetic diversity (Frankham *et al.*, 2002).

#### *2.4.3.1 Haplotypic diversity*

Genetic diversity of mitochondrial sequence data can be estimated at two levels, the nucleotide level and the haplotype level. A haplotype is defined as the constellation of alleles present at a particular region of the chromosome (Lincoln *et al.*, 1987), or in this thesis as an unique sequence of mtDNA. It is possible to measure the number of haplotypes within a population (*k*) (Nei, 1987). However, the haplotype number is difficult to compare among studies and populations, as the same sample size is required

for this to be meaningful. By calculating the haplotype diversity (*h* - also known as nucleon diversity), comparisons between studies can be made. Haplotype diversity is a function of the number and frequency of haplotypes within a sample without accounting for the relationships among haplotypes (Nei and Tajima, 1981). Haplotype diversity was calculated for the dugong in Australia to allow comparison between different studies and among the dugong demes around Australia (Chapter 4).

It is also possible to identify the extent of DNA variation at the nucleotide level. This approach is useful in practice as the size of the nucleon (segment of DNA) of interest varies. There are two measures of genetic diversity at the nucleotide level: Nucleotide diversity and nucleotide divergence. Nucleotide diversity ($\pi$) is the average number of nucleotide differences per site within a population (Nei, 1987). The nucleotide divergence is a similar measure being defined as the average number of substitutions per nucleotide site between populations (Nei and Tajima, 1983). Both the nucleotide diversity and nucleotide divergence were used in this study (see Chapter 4).

#### *2.4.3.2 Genotypic diversity*

Genotypic diversity based on nuclear DNA markers such as microsatellites, which are co-dominant markers that reveal copies of DNA, or alleles, from both parents. These markers are analysed following HWE models. Allelic diversity represents the total number of alleles in a population and as such is a simple measure of genetic diversity. A wide range of methods to assess genetic diversity are available. I focus on the relative proportions of heterozygotes in populations, following the methods of calculating $F_{st}$ (Wright, 1951) explained in section 2.4.4.

### *2.4.4 F-statistics and the design of population studies*

Population studies are usually structured to investigate groups within groups. For example, a region with five lakes, with each lake being a sub-population of the region, and schools of fish in the lakes being sub-populations within each lake. Refer to the groupings in the lake example above for the following explanation of the hierarchical structure of population studies. This hierarchical design allows for analyses to identify

the proportion of the total genetic differentiation attributable to the different levels (Hartl and Clark, 1989). One outcome of such analyses are the *F*-statistics, developed by Wright (1951) to describe the partitioning of genetic variation within and among subdivided populations. These statistics can allocate variability to three levels: the total population level, the subdivisions and the individuals within populations (Hedrick, 1985). For example, $F_{st}$ is a measure of the genetic differentiation between/among sub-populations (Hedrick, 1985). Phi ($\phi$) is an equivalent measure that is more appropriate for haploid markers and for comparison of these markers with codominant markers (Peakall and Smouse, 2005). For this reason $\phi$ statistics were used in this study for mitochondrial and microsatellite data. The subscripts following $\phi$ are indications of the level of variation that is being examined. In this study the subscripts 'st', 'sr' and 'rt' will be used. The subscript 'st' indicates the proportion of variance among populations relative to the total. With 'sr' indicating the proportion of variance among populations within regions and 'rt' the proportion of variance among regions relative to the total.

## 2.5 Molecular markers used for phylogeographic and population studies

### *2.5.1 Sequence data*

One commonly used approach to population and phylogeographic studies is the analysis of sequence data. This approach provides the most direct way of measuring genetic diversity. However, the cost of sequencing large numbers of samples is high, even though recent technical improvements have reduced the cost and time involved (Frankham *et al.* 2002).

#### *2.5.1.1 Mitochondrial DNA sequences*

Analyses of mitochondrial DNA (mtDNA) can be used in two areas for conservation: the first is the identification of Evolutionarily Significant Units (ESUs) and the second identification of Management Units (MUs) as suggested by Moritz (1994a). The concept of ESUs was developed to provide a basis for prioritising taxa for conservation, given resource limitations and that the existing taxonomy may not reflect genetic diversity (Moritz, 1994a). The purpose of assigning populations to an ESU is to ensure evolutionary heritage is recognised and protected. Recognition of ESUs is primarily

relevant to long term management and considers the pattern rather than the extent of divergence between populations/species (Moritz, 1994a). In contrast, MUs are defined by statistically demonstrable divergence in allele frequencies between populations in question, regardless of the phylogeny (Moritz, 1994b). Management units focus on the current population structure and are suitable for short-term management objectives (Moritz, 1994a).

Mitochondrial DNA is often used in studies of marine mammals for a number of reasons including its high rate of evolution, maternal inheritance, low effective population size and lack of recombination (Hoelzer *et al.*, 1998). The mtDNA is inherited maternally and is hence useful for identifying female-mediated gene flow in the population (Hoelzer *et al.*, 1998). This mode of inheritance lowers the effective population size four-fold compared with nuclear DNA (nDNA), allowing population differentiation to occur faster in mtDNA than nDNA (Hoelzer *et al.*, 1998). Haploid inheritance of the mitochondrial genome also means there is no recombination, therefore gene flow through evolutionary history, at least of the maternal lineage, can be examined (Hoelzer *et al.*, 1998).

The two regions of the mitochondrial genome most commonly used for studies on marine mammals are the mitochondrial control region and the cytochrome *b* gene. The mitochondrial control region is a non-coding segment of the mitochondrial genome that regulates the replication of this genome (described in further detail in section 3.1.1). The control region is commonly used due to its high level of variability (Brown *et al.*, 1986). The cytochrome *b* gene encodes one of the electron carrier proteins that make up complex III of the mitochondrial oxidative phosphorylation system (Hatefi, 1985). The positioning of the cytochrome *b* gene 5' of the control region in mammals has led to researchers using both together in numerous studies including some on marine mammals (see Appendix 1). This study utilises the mtDNA control region, which was found to contain the most variation in the entire mitochondrial genome of dugongs (section 3.3.1).

#### *2.5.1.2 Nuclear DNA sequences*

Alternatively, sequence data can be obtained from nuclear DNA (nDNA). The main advantage of using nDNA is that it is inherited bi-parentally. If the patterns produced from analysis of the mtDNA sequence data are different to those produced from nDNA, then there is an indication that sex-biased gene flow is occurring in the population (Prugnolle and de Meeus, 2002). Using nDNA does have its disadvantages, including recombination, multiple copies of genes and primer availability (Zhang and Hewitt, 2003). Nuclear genes normally have multiple alleles and in order to successfully obtain clear sequences, cloning of the polymerase chain reaction (PCR) product is necessary before sequencing. There are single-copy nDNA regions, which can be used preferentially to avoid the problem of duplicate genes. Single-copy nDNA contains a high level of historic genetic variation (Wan *et al.*, 2004) which would be useful for phylogeographic studies. However, single-copy nuclear DNA evolves 5 - 10 times slower than mtDNA (Wan *et al.*, 2004). For these reasons mtDNA was used preferentially over nDNA for the phylogeography section in this study (see Chapter 4).

### *2.5.2 Allele-based methods*

Many methods have been used to assess the genetic diversity in individuals and populations. Some of the more common procedures and markers are described below, including the advantages and disadvantages of each. The descriptions below have been taken from Frankham *et al.* (2002), unless otherwise specified.

#### *2.5.2.1 Allozymes*

A traditional method of identifying population differentiation that is visualised by gel electrophoresis. Allozymes, use the variation in size and electrophoretic charge of proteins, to quantify genetic diversity. One disadvantage of this method is that there is often a lack of variation, especially within small or bottlenecked populations.

#### *2.5.2.2 RAPD – random amplified polymorphic DNA*

Short random oligonucleotides are used as primer sequences to amplify fragments from DNA samples. These random primers yield a series of DNA fragments that are typically

separated on a poly-acrylamide gel. The genotypes are then scored based on a presence or absence of a particular band. That is, if there is a difference in the priming site for a sample no band will be amplified. The advantage of RAPDs is that many loci can be assayed without the need to sequence the genome and design specific primers. RAPDs can also be typed easily after non-invasive sampling. The main disadvantage with using RAPDs is the dominant mode of inheritance, meaning that heterozygotes cannot be distinguished. RAPDs also sometimes have poor repeatability. However, the longer the random primer that is used the greater the repeatability.

#### *2.5.2.3 AFLP – amplified fragment length polymorphism*

For this method genomic DNA is cut with a restriction enzyme and a synthetic adapter is ligated to the end of each fragment. A PCR is then conducted using a primer of the same sequence as the synthetic adapter. This method produces a presence/absence of bands as described for RAPDs. Similar advantages and disadvantages to those of RAPDs also apply to AFLPs. However, this procedure has considerably greater repeatability than RAPDs and is able to generate a greater number of loci for screening.

#### *2.5.2.4 RFLP – restriction fragment length polymorphism*

This method involves the digestion of genomic DNA with a restriction enzyme, and then the various bands are separated on a gel. The DNA is then transferred to a membrane and the membrane hybridised to a radioactive probe for the locus in question. The variation in DNA sequence at the restriction enzyme cut site is then evident from the different sized fragments, produced on the autoradiograph.

#### *2.5.2.5 VNTR – variable number tandem repeats*

VNTRs are also known as minisatellites and are representative of the original methods used for DNA fingerprinting. VNTRs are large, with the core repeat unit being between 10 and 100 bps. The considerable variation in minisatellites is produced by unequal crossing over – leading to different numbers of the repeat being present. Each individual in an outbreeding population will have a unique DNA fingerprint. The advantages of using VNTRs are the high variability and that nuclear variation is assessed over a wide

range of loci and no previous knowledge of the DNA sequence being typed is required. However the disadvantages include: individual loci are not normally identifiable, the inheritance of bands is not defined, and the amount of DNA required is great so these markers can not be used after non-invasive sampling (which usually yields only a small amount of DNA).

#### *2.5.2.6 SSR – simple sequence repeats or microsatellites*

Microsatellite loci are short tandem repeats of DNA (typically 2 - 6 bp in length). Microsatellites are highly variable in the number of repeats at each locus, resulting in variation in the length of the fragment. The most likely cause of this length variation is slippage during DNA replication, resulting in the addition or subtraction of repeat motifs (Schlötterer and Tautz, 1992). The main advantage of microsatellite loci over other markers is their high variability and co-dominant inheritance. These markers can be used to identify individuals (Richard *et al.*, 1996), populations on a variety of spatial scales (Allen *et al.*, 1995; Goodman, 1998; Hoelzel *et al.*, 1998; Slade *et al.*, 1998; Burg *et al.*, 1999; Gladden *et al.*, 1999) and trends across species (FitzSimmons *et al.*, 1995). The main disadvantage is that they need to be developed anew for each species. However, microsatellites developed for one species can often be used for closely related species (Beaumont and Bruford, 1999), as has been done in this study (Chapter 5). Microsatellites, identified for the Florida manatee (Garcia-Rodriguez *et al*., 2000) were utilised in this study to identify current population structure and gene flow in dugongs around Australia.

## 2.6 Phylogeographic studies on marine mammals

Most genetic studies on marine mammals are either phylogeographic or population genetic studies. Phylogeography is concerned with the principles and processes governing the geographical distributions of genealogical lineages (Avise, 1998). This approach results in an understanding of how populations have differentiated to their present state.

The mitochondrial control region was used in several studies on whales to identify genetic variation, with varying degrees of success depending on the spatial scale of interest. In humpback whales (*Megaptera novaeangliae*) the mitochondrial control region identified differentiation between populations on large scales, such as within the Southern Hemisphere and within ocean basins (Palsboll *et al.*, 1995; Baker *et al.*, 1998a), but failed to identify population structure in the sperm whale (*Physeter macrocephalus*) on a similar scale (Whitehead *et al.*, 1998). However, Lyrholm and Gyllensten (1998) did identify differentiation in the sperm whale using the mtDNA control region between ocean basins but not within the Atlantic Ocean. In contrast, phylogeographic studies on seals and sea lions using the mitochondrial control region always showed some level of differentiation within ocean basins (Maldonado *et al.*, 1995; Bickham *et al.*, 1996; Stanley *et al.*, 1996; Slade *et al.*, 1998; Burg *et al.*, 1999) and between oceans (Stanley *et al.*, 1996).

The cytochrome *b* gene has been shown to be a useful marker in studies on seals and sea lions, and some whales (Lento *et al.*, 1994; Maldonado *et al.*, 1995; Yoshida and Kato, 1999). Yoshida and Kato (1999) used the mtDNA control region and Cytochrome *b* to identify differences in the Brydes' whale (*Balaenoptera edeni*) between different ocean basins. Lento *et al.* (1997) used only cytochrome *b* gene to identify the differences between the New Zealand and Western Australian rookeries in the New Zealand fur seals (*Arctocephalus forsteri*).

One problem with phylogeographic studies such as those above, is the expense involved with sequencing numerous individuals, and the need to use more than one locus for a robust and conclusive study. A number of studies on marine mammals have combined a marker that can be used for phylogeography studies with one commonly used in population genetics (Slade *et al.*, 1998; Burg *et al.*, 1999). Studies that use more than one marker system are more likely to identify genetic differentiation of the population in question (Appendix 1).

## 2.7 Population genetic studies on marine mammals

There are several methods used in population genetic studies as outlined above. Here I will look at specific examples using marine mammals and identify and evaluate commonly used markers and methods. As equipment becomes more advanced so too does the methodology for population studies.

Numerous studies were conducted on marine mammals in the late 1980's and early 1990's using allozymes. These include studies on long and short finned pilot whales (*Globicephala melaena* and *G. macrorhynchus*) (Anderson, 1988; Wada, 1988) and bottlenose dolphins (*Tursiops truncatus*) (Duffield and Wells, 1991; Goodwin *et al.*, 1996). The study on bottlenose dolphins (*T. truncatus*) by Goodwin *et al.* (1996) failed to come to any conclusive decision about population structure on the east coast of South Africa. A cluster analysis conducted on the data showed a grouping of the northern and southern dolphins, however the $F_{st}$ statistics failed to identify significantly different populations along the coast. Contrasting this, studies by Wada (1988) and Anderson (1988) both found significant differences between the populations of pilot whales (*G. melaena* and *G. macrorhynchus*) studied on a similar spatial scale, off the coast of Japan and the Faroe Islands respectively. These most likely reflect geographic differences in the species' ranges.

Studies on seals, dolphins and porpoises have given mixed results using RFLPs. Garcia-Martinez *et al.* (1995) looked at the differentiation of the striped dolphin (*Stenella coeruleoalba*) along the Spanish Mediterranean coast and failed to identify genetic structure. However, studies on larger spatial scales have shown divergence between populations. For example, the study by Garcia-Martinez *et al.* (1999), showed a significant differentiation of striped dolphins (*S. coeruleoalba*), with no genotype being shared between the Mediterranean and Atlantic coasts. Dowling and Brown (1993) also identified population structure between the Atlantic and Pacific populations of bottlenose dolphins (*T. truncatus*). Similarly, Wang *et al.* (1996) used RFLPs to identify population structure of the harbour porpoise (*Phocoena phocoena*) in the North Atlantic. The ability of RFLPs to identify population structure is not restricted to

dolphins with Boskovic *et al.* (1996) showing a similar lack of shared genotypes between the West and East North Atlantic Ocean for grey seals (*Halichoerus grypus*).

The use of minisatellites allowed the identification of relatedness between individuals in harbour seals (*Phoca vitulina*) (Schaeff *et al.*, 1999). Minisatellites have also been used to attempt to distinguish populations in the harbour seal (Kappe *et al.*, 1995), the genetic variation in beluga whale (*Delphinapterus leucas*) (Patenaude *et al.*, 1994), and large-scale differences between the North and South Atlantic right whales (*Eubalaena glacialis* and *E. australis*) (Schaeff *et al.*, 1997). Results of these studies are varied with a distinct difference being noted in the right whales but not in the smaller scale study between the Dutch Wadden Sea and Scottish populations of the harbour seal (Kappe *et al.*, 1995) (Appendix 1).

Contrary to some of the other markers discussed here, the studies using microsatellites all showed population differentiation at a number of scales, regardless of the species. These scales ranged from between breeding sites in Britain (Allen *et al.*, 1995), to within oceans and regions in oceans (Hoelzel *et al.*, 1998; Slade *et al.*, 1998; Gladden *et al.*, 1999) to between oceans (FitzSimmons *et al.*, 1995). This pattern was consistent even when a number of markers were used. For example microsatellites were used in conjunction with RFLPs to identify population structure in the Atlantic walrus (*Odobenus rosmarus rosmarus*) (Andersen *et al.*, 1998).

From examining various methods and studies it becomes apparent that the most useful approach to take when studying marine mammals is a combination of phylogeographic and population genetic markers. The microsatellite markers appeared to provide the greatest power to differentiate populations on a number of scales. Any of the above mentioned methods would possibly provide the required differentiation if the study was concerned with only large-scale geographic differences. However, if the study is to have multiple scales, as is the case with my study, then microsatellites appear to be the most useful approach. Using microsatellites in combination with mitochondrial sequence data will also provide further information and possible differentiation. Therefore, I have used

a combination of mtDNA sequencing (Chapters 3 and 4) and microsatellite loci (Chapter 5) to gain an understanding of the population structure of dugongs.

## 2.8 Potential problems with molecular studies on marine mammals

A molecular study on a threatened marine mammal, such as the dugong, faces many potential difficulties. The most obvious one is the collection of samples. As dugongs inhabit shallow coastal, often turbid, water the ability of researchers to collect samples from live animals is limited. Therefore, in my study a large number of samples were collected from stranded, dead dugongs, or dugongs that were hunted by Indigenous communities. Thus sampling was largely opportunistic. Samples from animals in various stages of decay, from freshly dead to mummified carcasses, resulted in difficulties in extracting DNA (due to poorly preserved samples) and difficulties in PCR (due to degraded DNA). Thus limiting the number of samples able to be included in final analyses.

## 2.9 How dugongs compare to other marine mammals

### *2.9.1 Life history similarities*

The biology of dugongs is comparable to that of other marine mammals, with the life span being approximately 70 years (Marsh, 1995). The amount of time and energy invested in reproduction by dugongs is high, and similar to other marine mammals, with the gestation period being 13 months, the calf suckling for approximately 18 months and the interval between calving ranging from 3 - 7 years (Marsh, 1995). The mating strategies appear to vary on either side of Australia, as mentioned previously (section 2.4.1), which is an important difference between dugongs and other marine mammals. The cumulative effects of these life history traits in dugongs is a slow-growing population and with long generation times.

### *2.9.2 Evolutionary history of Sirenia*

Although similar to cetaceans in life history, sirenians differ in evolutionary history. Marine mammals arose from terrestrial species in two groups (Heyning and Lento, 2002). The pinnipeds (seals, sea lions and walruses) and the sea otters arose from the

Order Carnivora which includes lions, tigers and bears (Heyning and Lento, 2002). The other terrestrial group is the Ungulata, which includes the subgroups Artiodactyla and Paenungulata (Heyning and Lento, 2002). The Cetacea (whales and dolphins) are most closely related to the two-toed ungulates (the Artiodactyla) including sheep, camels, hippos and pigs (Heyning and Lento, 2002). Recent molecular studies have suggested that cetaceans have strong evolutionary ties with the Artiodactyla prompting a name change to the Cetartiodactyla by O'Leary (1999). Sirenia however, are in a super-ordinal grouping called the Paenungulata. The Paenungulata was first proposed by Simpson (1945) when he grouped Sirenia with the Proboscidea (elephants), the Hyracoidea (hyraxes), and a number of extinct fossil orders. Molecular studies have verified the presence of the Paenungulata and place it within the Afrotheria (also within the Ungulata) which includes the aardvark, golden moles and tenrecs (Stanhope *et al.*, 1998).

## 2.10 Previous molecular studies on Sirenia

The genetic variability of members of the order Sirenia is under-studied. Two published studies have been conducted on West Indian Manatees (*Trichechus manatus*). McClenaghan and O'Shea (1988) investigated the allozyme variation in the Florida Manatee (*T. m. latirostris*). These authors found little differentiation between geographic regions around Florida. A more recent study using mtDNA sequence data of the West Indian Manatee (*T. manatus*) indicated strong patterns of population differentiation among most of the populations examined (Garcia-Rodriguez *et al.*, 1998). This study also identified three maternal lineages among manatees, suggesting deep phylogeographic separation which has only recently been bridged (Garcia-Rodriguez *et al.*, 1998). A third published study on the Amazonian manatee (*T. inunguis*) by Cantanhede *et al.* (2005) also used the mtDNA control region. Cantanhede *et al.* (2005) suggested that the Amazonian manatee acts as a panmictic population and contains relatively high genetic diversity.

Results from a number of unpublished studies on manatees have been presented at international conferences. A total of 470 bps of the mtDNA control region was sequenced from 62 samples of the West Indian manatee from Puerto Rico by

Rodriguez-Lopez *et al.* (2003). They found two variable sites and three haplotypes. On the basis of this, two management units for the manatee in Puerto Rico were suggested. Vianna *et al.* (2003) conducted a phylogeographic study on the manatee species and found eight animals that could be hybrids between the West Indian manatee and the Amazonian manatee based on the mitochondrial control region sequences. These hybrids were confirmed by examination at two microsatellite loci (Vianna *et al.*, 2003).

Parr (2000) conducted a molecular study on the relationships between Sirenia and the other Paenungulates and the Aardvark by analysis of a 648 bp fragment of mtDNA including 125 bp of the cytochrome b gene, the tRNA threonine and tRNA proline and a fragment of the control region. Parr's results are summarised in section 3.4.4.

Tikel (1997) suggested, by conducting analyses of the mtDNA control region, the presence of two mitochondrial lineages among Australian dugongs. Tikel (1997) had too few samples from west of Torres Strait and from Western Australia to permit conclusions to be reached about the penetration of the east coast lineage into that area. A study by Palmer *et al.* (2003) found two distinct maternal lineages within Thailand dugongs that overlapped in geographical distribution, based on mtDNA control region sequences of 40 dugongs. Palmer *et al.* (2003) suggested that these lineages are a result of philopatry in dugongs.

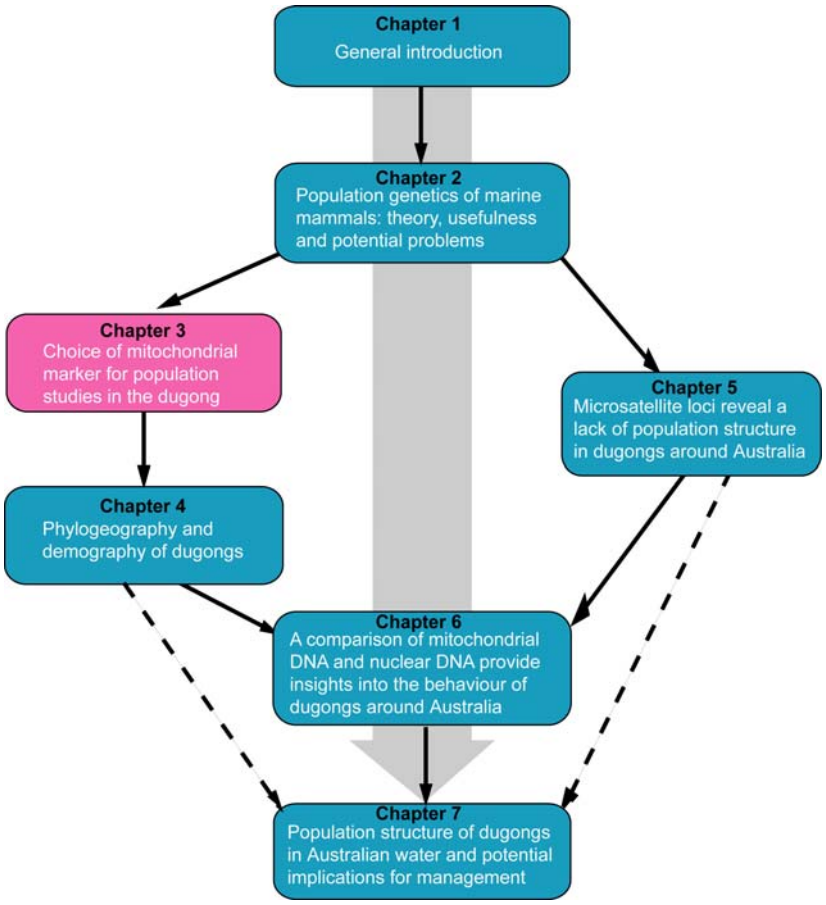
### *2.10.1 Conclusions*

This chapter has described the usefulness of mtDNA and nDNA when used in combination to identify the population structure of marine mammals. Therefore, I used a portion of the mtDNA control region shown to have a high level of genetic variation within dugongs (Chapter 3) to assess the phylogeography of dugongs around Australia (Chapter 4). Nuclear DNA markers in the form of microsatellite loci were used to identify the current levels of gene flow among dugong populations in Australia, and between Australia and other countries (Chapter 5). The importance of understanding the biology and ecology of organisms that may not fit the assumptions associated with many population-genetic theories, is discussed in relation to dugongs (Chapter 5). A

direct comparison of the outcomes of these two marker systems is made (Chapter 6). These studies provide insight into the genetic diversity of the dugong around Australia.

# Chapter 3: Choice of mitochondrial marker for population studies in the dugong

*A common marker for population studies is DNA sequence data generated from the mitochondrial genome in animals. Comparisons of the two complete mitochondrial genomes of the dugong available in GenBank showed most variation between the genomes to be clustered in the control region and in the ND5 gene. The control region was therefore chosen as the marker for this phylogeographic study. In this chapter the 'anatomy' of the control region is characterised and the variation within the control region to be found at different phylogenetic levels (among dugongs, among sirenians and among paenungulates) was explored. This study is the first to use a complete sequence of the Florida manatee control region (*Trichechus manatus*) and the first to sequence any part of the tree hyrax (*Dendrohyrax dorsalis*) control region.*



## 3.1 Introduction

Mitochondrial DNA (mtDNA) is often used for phylogeographic studies in marine mammals as described in section 2.6. I therefore compared the two complete dugong mitochondrial genomes, available in GenBank, to identify which regions may be appropriate for population studies within the dugong.

### *3.1.1 Animal mitochondrial genome and the control region*

The mammalian mitochondrial genome is circular, ranging in size from 14 - 19 kb (Gillham, 1994). The complete dugong mitochondrial genome is 16.85 kb in length (Arnason *et al.*, 2002). The two strands of the mtDNA genome are known as the Heavy (H) and Light (L) strands. The H-strand codes for most of the proteins and RNAs in the mitochondrion (16s rRNA, 12s rRNA, 14 tRNAs, the three largest subunits of cytochrome *c* oxidase, cytochrome *b*, two subunits of ATP synthase and seven subunits of NADH dehydrogenase). The L-strand includes coding sequences for eight tRNAs and one subunit of NADH dehydrogenase (ND6) (Gillham, 1994). Mitochondrial genes lack introns, and the genes on the H-strand are separated from each other by a few nucleotides at most (Gillham, 1994). The control region is the only substantial non-coding region of the mtDNA. This stretch of DNA contains the origin of H-strand replication (OH) and promoters for H- and L-strand transcription (Gillham, 1994; Sbisa *et al.*, 1997).

The following description of mtDNA replication below is paraphrased from Walberg and Clayton (1981) and Gillham (1994). The OH acts as a primer sequence by initiating the synthesis of a daughter H-strand that then produces a triplex d-loop structure. The daughter H-strand synthesis proceeds in a unidirectional manner until completion. However, the L-strand synthesis begins when the replication of the H-strand is approximately 67% complete. At this stage, the L-strand origin of replication is exposed by the separation of the two original DNA strands and synthesis of the L strand begins. With the OH being situated in the control region it is obvious that this section of non-coding mtDNA plays an important role in the replication of the mtDNA. Perhaps because of this, the control region in all mammalian species studied so far appears to have similar conserved features.

However, portions of the control region lying outside these functional conserved regions are highly variable in terms of nucleotide substitutions, short insertions/deletions and dynamics of variable number tandem repeats (Brown *et al.*, 1986; Sbisa *et al.*, 1997; Randi and Lucchini, 1998). This variability allows the mtDNA control region to be used to study, for example, phylogeography, demographic history, identification of secondary contact between lineages, taxonomic status, and forensic applications (Frankham *et al.*, 2002). Examples of studies that have utilised the control region in marine mammals are given in section 2.6.

### *3.1.2 Sirenia mtDNA studies to date*

To date, the mitochondrial DNA of sirenians has been used in only a small number of studies, mostly investigating the phylogenetic position of sirenians in relation to the evolution of placental and eutherian mammals (Springer and Kirsch, 1993; Springer and Douzery, 1996; Stanhope *et al.*, 1998). These studies have utilised the cytochrome *b* gene, tRNA-valine, the 12S rRNA, and the 16S rRNA regions from, usually, a single individual of each species. Other studies that have used the mitochondrial DNA of sirenians were to demonstrate the usefulness of universal primers over a broad range of taxa (Verma and Singh, 2003) and the usefulness of non-invasive sampling in sirenians (Tikel *et al.*, 1994).

An initial study of the mitochondrial control region of the dugong was conducted by Tikel (1997) who sequenced the entire control region (1268 bp) from two individuals. Tikel (1997) noted that 84% (*i.e.* 39 variable sites) of the differences between the two dugong sequences occurred in the first 194 bp. A phylogeographic study on the West Indian Manatee used the first 410 bp of the mitochondrial control region where 51 polymorphic sites were identified (Garcia-Rodriguez *et al.*, 1998). Similarly, a recent study on the phylogeography of the Amazonian manatee used the first 361 bp of the control region and identified 61 variable sites in American trichechids (Cantanhede *et al.*, 2005). Unpublished studies on the phylogeography of sirenians using the control region include two studies on manatees and one on dugongs, all utilised the 5' end of the mitochondrial control region (Palmer *et al.*, 2003; Rodriguez-Lopez *et al.*, 2003; Vianna *et al.*, 2003). The only other study on the control region of Sirenia was by Parr

(2000) who investigated the intra and inter-specific relationships of the three extant species of manatees using a 648 bp fragment including a 398 bp section at the 5' end of the control region.

### *3.1.3 Sirenians and paenungulates*

In order to assess the usefulness of the control region as a molecular marker for phylogeographical studies, the ability of this region to recover accepted phylogenies was examined by analysis of the superorder Paenungulata.

The classification of Sirenia and their relatives, based on morphology, has always been controversial. The superorder Paenungulata was proposed by Simpson (1945) as indicated in section 2.9.2. However, Simpson (1945) referred to the possibility that the Hyracoidea may in fact have a closer relationship with the Perissodactyla. This hypothesis was later supported by a morphological study conducted by Fischer (1989). The Hyracoidea-Perissodactyla clade was also supported by Fischer and Tassy (1993) and Prothero (1993). However, evidence from other morphological studies contradicts the grouping of Hyracoidea and Perissodactyla, and supports grouping Hyracoidea with the Tethytheria (Proboscidea + Sirenia) (Rasmussen *et al.*, 1990; Shoshani, 1993).

Molecular studies thus far strongly support the recognition of the Paenungulata as proposed by Simpson (Kleinschmidt *et al.*, 1986; Lavergne *et al.*, 1996; Liu and Miyamoto, 1999; Springer *et al.*, 1999), and of the Afrotheria which includes the Paenungulata, aardvarks, tenrecs and elephant shrews (Porter *et al.*, 1996; Liu and Miyamoto, 1999; Springer *et al.*, 1999; Murphy *et al.*, 2001; Murata *et al.*, 2003). An early molecular phylogenetic study comparing the haemoglobin sequences of a wide range of mammalian species found a monophyletic Paenungulata (Kleinschmidt *et al.*, 1986). Lavergne *et al.* (1996) used the 12S rRNA sequences of 11 eutherian orders including two species of Sirenia, two proboscideans and two hyraxes. Phylogenetic analyses indicated high support for the monophyly of each of the paenungulate orders and the Paenungulata clade (Lavergne *et al.*, 1996). Similarly the phylogenetic analyses of the Interphotoreceptor Retinoid Binding Protein (IRBP) also strongly supported the Paenungulata clade (Stanhope *et al.*, 1996).

### *3.1.4 Aim of this chapter*

A large amount of variation occurring within the control region of the dugong mitochondrial DNA was identified from comparison of the complete mitochondrial genome from two individuals. This region is therefore explored further to ascertain its usefulness in phylogenetic and demographic studies of the dugong. First, the structure of the control region was described. Then the amount of variation found at the different classification levels (among dugongs, among sirenians and among paenungulates) was explored. The phylogenetic relationship among the paenungulate orders is inferred based on the mitochondrial control region. I then assess the usefulness of the control region for phylogeographic studies on the dugong.

## 3.2 Methods

### *3.2.1 Species and samples used in this study*

The two published mitochondrial genomes of dugongs available in GenBank were aligned in Sequencher (GeneCodes, 1991) to allow the identification of polymorphic sites. The genes, and other features, where polymorphic sites resided were identified from the GenBank annotations. The greatest number of polymorphic sites was identified in the control region. Therefore this region was looked at in further detail.

The complete control regions of four individual dugongs (*Dugong dugon*), three manatees (*Trichechus manatus*) and partial control region of the tree hyrax (*Dendrohyrax dorsalis*) and rock hyrax (*Procavia capensis*) were sequenced. The dugong samples include individuals from Torres Strait (MD56), Townsville (B61), Ashmore Reef (SW1) and the Philippines (LEM1). Additional sequences from a Moreton Bay dugong (D3) and a Torres Strait (T677) individual were from Tikel (1997). Two complete control region sequences, GB1 and GB2, were obtained from GenBank (Table 3.1). Robert Bonde kindly provided the manatee tissue. A consensus sequence of three manatees was used in these analyses due to the high similarity between samples and the difficulty of sequencing all sections of all three individuals. The DNA of the tree hyrax (*D. dorsalis*) and the rock hyrax (*P. capensis*) was provided by G. Bernardi and O. Madsen respectively. The tree hyrax DNA was from tissue number T-0512 of the tissue collection of mammals at Montpellier. Murata *et al.* (2003)

published the complete mitochondrial control region of *P. capensis* which was also used in this study. The control region sequences of the African elephant (*Loxodonta africana*) (Hauf *et al.*, 2000) and Asian elephant (*Elephas maximus*) were obtained from GenBank. Two members of the Afrotheria, the aardvark (*Orycteropus afer*) and the cape golden mole (*Chrysochloris asiatica*) were used as outgroups for the phylogenetic part of the study. See Table 3.1 for accession numbers.

**Table 3.1** Sequences from GenBank included in the analyses. The species, accession numbers and sequence lengths are indicated. * Partial control region sequence only.

| Common Name | Scientific Name | Accession Number | Sequence Length (bp) |
|---|---|---|---|
| African Elephant | *Loxodonta africana* | NC_000934 | 1 448 |
| Asian Elephant | *Elephas maximus* | NC_005129 | 1 411 |
| Dugong | *Dugong dugon* | NC_003314 | 1 419 |
| | | AY075112 | 1 415 |
| Caribbean Manatee | *Trichechus manatus* | AF046160 | 410* |
| Amazonian Manatee | *Trichechus inunguis* | AF046159 | 410* |
| Rock Hyrax | *Procavia capensis* | NC_004919 | 1 284 |
| Aardvark | *Orycteropus afer* | NC_002078 | 1 363 |
| Cape Golden Mole | *Chrysochloris asiatica* | NC_004920 | 1 233 |

### *3.2.2 DNA extraction, amplification and sequencing*

Genomic DNA extraction of tissue from dugong, and Florida manatee (*Trichechus manatus manatus*) followed van Oppen *et al.* (1999). This protocol involved the digestion of small amounts of the epidermis with SE buffer (0.075 M NaCl, 0.025 M EDTA, 0.5% SDS) and proteinase K at 37°C overnight. A high concentration of salt and chloroform was then used to extract the proteins from the digested tissue. Precipitation of the genomic DNA was done with 100% isopropanol, followed by two washes with 70% ethanol to remove any additional salt that may have precipitated. The remaining DNA pellet was air-dried and resuspended in TE (10 mM Tris, 1 mM EDTA).

Primer sequences used in the amplification and sequencing of the control region from dugongs, hyraxes and manatees were designed by Kocher *et al.* (1989), Tikel (1997) or myself (Table 3.2). The mtDNA control region in all species was amplified in three segments using a combination of different primers (A24 and A58; A77 and A80; A78 and A26) outlined in Table 3.2. The positions of the primers along the control region are

indicated on Figure 3.1. Amplification was done using Qiagen *Taq* PCR reagents (Qiagen catalogue number 201223) in the following concentrations per 25 µL reaction: 1 x PCR buffer, 2 mM $MgCl_2$, 0.16 mM dNTPs, 1 x Q solution, and 1 unit of *Taq*. The following PCR profile was used: an initial step of 5 minutes at 96ºC followed by 30 cycles of 30 secs at 96ºC, 30 secs at 50ºC, 1 minute at 72ºC, followed by a final step of 10 minutes at 72ºC. For the manatee and hyrax samples, 2 x BSA was added to the PCR reaction instead of the 1x Q solution and the annealing temperature was raised to 54ºC.

**Table 3.2** List of primers used to amplify the control region of dugong, manatee and hyrax. Sequences for plasmid primers (U19 and T7), used when sequencing clones, are also listed. Refer to Figure 3.1 for placement of primers within the control region. $T_m$ = melting temperature, $T_{annealing}$ = annealing temperature PCR performed at. Primers with two annealing temperatures indicate different annealing temperatures required for different species.

| Primer | Sequence | $T_m$ | $T_{annealing}$ | Reference |
|---|---|---|---|---|
| A24 | TCA AAG CTT ACA CCA GTC TTG TAA ACC | 76 | 50 | Kocher* |
| A26 | TAA CTG CAG AAG GCT AGG ACC AAA CCT | 78 | 54 | Kocher* |
| A77 | CGA GAA ACC AGC AAC CCG C | 62 | 50, 54 | Tikel** |
| RA77 | GCG GGT TGC TGG TTT CWC G | 55 | | McDonald*** |
| RA78 | GTC CTC GAG CAT TGA CTG | | 50 | McDonald*** |
| A78 | CAG TCA ATG CTC GAG GAC | 56 | 54 | Tikel** |
| A58 | CCT GAA GTA RGA ACC AGA TGT C | 66 | 50 | Tikel** |
| A80 | CCC GTA CCC TTA CTT TCT G | 58 | 54 | Tikel** |
| Rock Hyrax1 | AGA TGY CAG RTA TAG ATY CAG | 49 | 50 | McDonald*** |
| TreeHyraxA77 | CGW GAA ACC AGC AAC CCG CCA C | 60 | 50 | McDonald*** |
| HyraxCytb | CCG ACA AAA TCC CAT TTC AC | | | McDonald*** |
| U19 | GTT TTC CCA GTC ACG ACG T | - | - | |
| T7 | TAA TAC GAC TCA CTA TAG GC | - | - | |

*Designed by Kocher *et al.* (1989)
**Designed by Tikel (1997)
***Designed by author

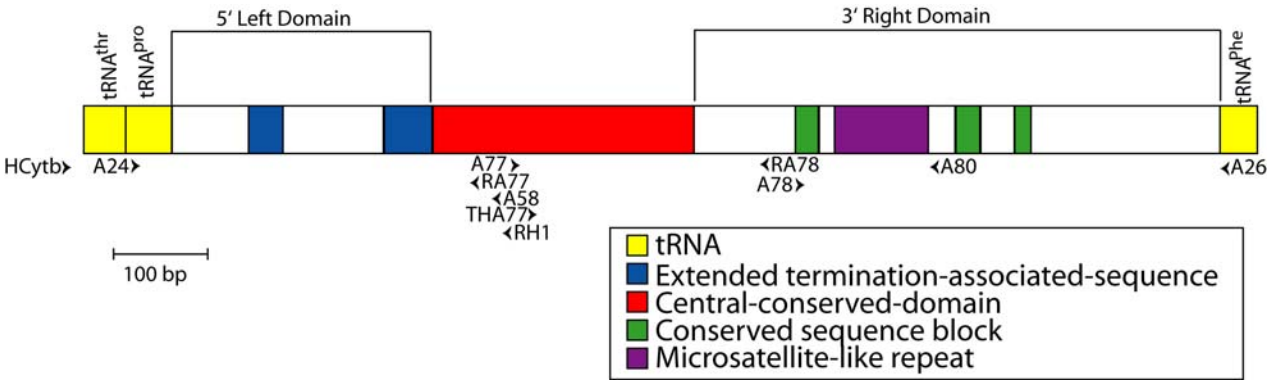



**Figure 3.1** Schematic diagram of the structure of the dugong control region. Positions of primers along the dugong control region are identified.

Direct sequencing was performed on the dugong PCR products. However the manatee and hyrax PCR products were cloned due to the presence of multiple bands. A pMOS-blue blunt end cloning kit (Amersham Bioscience) was used as per the manufacturer's instructions. White colonies were then picked and a PCR using the plasmid primers U19 and T7 was conducted (Table 3.2). A single clone containing the correct size insert was sequenced. The complete control region sequence for the tree hyrax and the rock hyrax could not be obtained. Additional primers outside of the control region (designed from GenBank sequences of hyrax species) and within the control region were designed (Rhyrax1, TreeHyraxA77, HyraxCytb - Table 3.2) but these were not successful in producing usable products.

For sequencing, PCR products were excised from a 1% 1x TAE (40 mM Tris-acetate, 1 mM EDTA) agarose gel and purified using a QIAquick gel purification kit (Qiagen Catalogue Number 28704) following manufacturer's instructions. DNA sequencing used ABI Big Dye chemistry (Applied Biosystems catalogue number 430315509197). Sequences were cleaned with a column of G-50 fine sephadex (Amersham Bioscience), and run on an ABI377 sequencer. Sequences were visualised and checked using Sequencher 3.1.1 (GeneCodes, 1991) and aligned in Se-Al v1.0a1 (Rambaut, 1996).

### *3.2.3 Characterising the dugong mitochondrial control region*

The eight complete dugong control region sequences were aligned in Se-Al v1.0 (Rambaut, 1996). The characterisation of the control regions of dugongs was then undertaken with reference to the conserved sequence blocks and regions outlined in Sbisa *et al.* (1997). This process included locating and annotating the extended termination-associated-sequences (ETAS) 1 and 2, the central-conserved-domain (CCD), and the three conserved sequence blocks (CSB). PAUP v4.0b10 (Swofford, 2000) was used to calculate, from the dugong control regions, the number of variable sites, number of transitions and transversions, and the base composition of each domain.

Traditionally, characterisation of the control region of vertebrates includes reference to various secondary structures that can be produced from the given sequences. Brown *et al.* (1986) outlined the possibility of significant secondary structuring within certain

areas of the control region as having a possible regulatory function. These areas, namely ETAS 1 and 2, the CSBs and the microsatellite repeat, identified in the dugong were analysed for the potential for secondary structure formation using Mfold (Zuker, 1996) available on a server at the Macfarlane Burnet Centre. Sequences were treated as RNA or DNA. Sequences were treated as RNA as the H-strand synthesis is initiated by RNA priming (Gillham, 1994). Folding temperature was set at 37°C, with 5% sub-optimality. This sub-optimality measure means only foldings within 5% from the minimum free energy will be computed. The 5' left domain and the 3' right domain were also analysed for production of secondary structures. Secondary structures that are reported were those produced when sequences were treated as DNA; the structures produced when treated as RNA were similar. In order to compare the secondary structures produced from the dugong ETAS regions, analyses were conducted, as above, on the ETAS regions identified by Sbisa *et al.* (1997).

The uncorrected 'p' pairwise distances among dugongs was calculated in PAUP v4.0b10 (Swofford, 2000). The distance measures were obtained for different portions of the control region: the entire control region, the three domains of the control region and the conserved motifs (ETAS 1 and 2, CSB 1, 2 and 3) separately.

### *3.2.4 Sequence alignment and characterisation of paenungulate control regions*

Additional control region sequences were obtained from GenBank (Table 3.1). The sequences were first aligned using ClustalX (Thompson *et al.*, 1997) then checked visually in Se-Al v1.0a1 (Rambaut, 1996) and the alignment adjusted if necessary. The alignment of the entire control region of the paenungulates was 1637 bp long (including insertions and/or deletions (*i.e.* indels) and the microsatellite region). This alignment is hereafter referred to as 'alignment one'. An additional alignment of the Paenungulata was also produced, including the most conserved potions of the region but excluding areas of many indels, and variable sections greater than 3 bp that were not alignable across all taxa. The regions excluded from this alignment were mainly those at either end of alignment one. The final conserved alignment of the paenungulates was 857 bp

long, which also excluded the microsatellite repeat. This alignment is hereafter referred to as 'alignment two'.

Comparisons with the features in the dugong control region facilitated locating and annotating the extended termination-associated-sequences (ETAS) 1 and 2, the central-conserved-domain (CCD), and the three conserved sequence blocks (CSB) in other paenungulates. The pairwise distances among all paenungulates were calculated in PAUP v4.0b10 (Swofford, 2000) using the uncorrected 'p' pairwise distance measure. The distance measures were obtained for different portions of the control region: the entire control region, the three domains of the control region and the conserved blocks (ETAS 1 and 2, CSB 1, 2 and 3) separately.

### *3.2.5 Substitution saturation tests and phylogenetic analyses*

A test for substitution saturation within the paenungulates (on both alignment one and alignment two) and dugongs was conducted using DAMBE (Xia and Xie, 2001; Xia *et al.*, 2003). Results reported are for tests where the proportion of invariable sites was set to 0.07317 as suggested from Modeltest (Posada and Crandall, 1998). This result is a conservative estimate of the proportion of invariable sites for the dugong alignment. An estimate of the proportion of invariable sites suggested from Modeltest (Posada and Crandall, 1998) for paenungulate alignment one was zero. The substitution tests were conducted using both these estimates. The significance levels of the results were not changed with the different proportions of variable sites. This test suggested that the paenungulate alignment two was most appropriate for phylogenetic analyses. Therefore, maximum parsimony analysis and maximum likelihood analyses [using the substitution model HKY85+G as suggested from Modeltest (Posada and Crandall, 1998)] were conducted in PAUP v4.0b10 (Swofford, 2000) on the Paenungulata alignment two.

## 3.3 Results

### *3.3.1 Comparison of the entire mitochondrial genome of dugongs*

A total of 173 polymorphic sites were identified between the two complete mitochondrial genomes (Table 3.3). This comparison identified the control region as

having the most polymorphic sites (45) followed by the ND5 gene (23; Table 3.3). The control region was then chosen for further study. Analyses of these two complete control region sequences from GenBank (GB1 and GB2) showed that they are representative of the two mitochondrial lineages of dugongs within Australia (Chapter 4); therefore they should provide a good indication of the amount of variation within dugongs around Australia. However, due to the lack of information on the origin of samples they were not included in the phylogeographic study (Chapter 4).

**Table 3.3** The number of polymorphic sites (including indels) within each identifiable region of the two complete mitochondrial genomes of dugongs. The base position numbers and names of each region are taken from GenBank accession number NC_003314. The genome sequence that was compared is GenBank accession number AY075112. Identical tRNAs between the two genomes have not been included in the table.

| Bp | Gene | Number of polymorphic sites |
|---|---|---|
| 70 – 1026 | 12s rRNA | 6 |
| 1095 – 2659 | 16s rRNA | 6 |
| 2660 – 2734 | tRNA-Leu | 1 |
| 2735 –3691 | ND1 | 7 |
| 3903 – 4946 | ND2 | 15 |
| 4945 – 5012 | tRNA-Trp | 2 |
| 5016 – 5084 | tRNA-Ala | 1 |
| 5259 – 5325 | tRNA-Tyr | 1 |
| 5327 – 6868 | COX1 | 10 |
| 6871 – 6939 | tRNA-Ser | 1 |
| 6947 – 7015 | tRNA-Asp | 2 |
| 7016 – 7699 | COX2 | 5 |
| 7702 – 7768 | tRNA-Lys | 1 |
| 7769 – 7972 | ATP8 | 1 |
| 7930 – 8616 | ATP6 | 5 |
| 8610 – 9393 | COX3 | 4 |
| 9463 – 9808 | ND3 | 2 |
| 9879 – 10175 | ND4L | 2 |
| 10169 – 11546 | ND4 | 14 |
| 11616 – 11674 | tRNA-Ser | 1 |
| 11675 – 11740 | tRNA-Leu | 2 |
| 11745 – 13556 | ND5 | 23 |
| 13553 – 14080 | ND6 | 7 |
| 14150 – 15294 | CytB | 9 |
| 15431 – 16850 | Control Region | 45 |
| | OVERALL | 173 |

### *3.3.2 Structural arrangement and characterisation of the control region of dugongs*

As has been observed in other mammals, three domains can be identified in the control region of dugongs, 5' left domain, the central conserved domain and the 3' right

domain. The complete dugong control regions sequenced in this study range in length from 1 402 to 1 468 base pairs. The sequences obtained from GenBank (GB1 and GB2) are 1 419, and 1 415 bp in length, whereas the sequences from Tikel (1997) are shorter (both T677 and D3 are 1 268bp). This variation between individuals results from the presence of variable numbers of a tandem microsatellite repeat in the 3' right domain positioned between CSB1 and CSB2 (Figure 3.1).

Of 41 variable sites found in an alignment of the eight complete dugong control region sequences (Appendix 2), 29 were in the 5' left domain (total length of 350 bp), three in the central conserved domain (total length of 207 bp) and nine in the 3' right domain (total length of 920 bp), excluding differences in the number and composition of tandem repeats in the microsatellite region. The variable sites were made up of 25 T/C substitutions, ten G/A substitutions, two G/C substitutions (*i.e.*, 35 transitions and two transversions) and four indels each of a single base (Table 3.4). About 70% (70.3%) of the total variability of the control region occurs in the first 277 base pairs.

**Table 3.4** Table of base substitutions (excluding the four indels) within the dugong control region calculated from the sequence alignment shown in Appendix 2.

| | **T** | **C** | **G** | **A** |
|---|---|---|---|---|
| **T** | - | | | |
| **C** | 25 | - | | |
| **G** | 0 | 2 | - | |
| **A** | 0 | 0 | 10 | - |

Although the variability differs between the domains, analyses indicate that the base frequency among domains is similar, with the entire control region being G-poor (Figure 3.2). The 5' left domain has the greatest frequency of T, whereas the 3' right domain has the greatest proportion of A (Figure 3.2).

The structural features of the mammalian control region described in Sbisa *et al.* (1997) were identified by alignment of the dugong sequences with those published (Figure 3.3). These features include ETAS 1 and ETAS 2, and the conserved sequence blocks 1,

2 and 3 (Figures 3.1, 3.3 and Appendix 3). All features previously described in mammalian control regions could be identified in the dugong by sequence similarity.

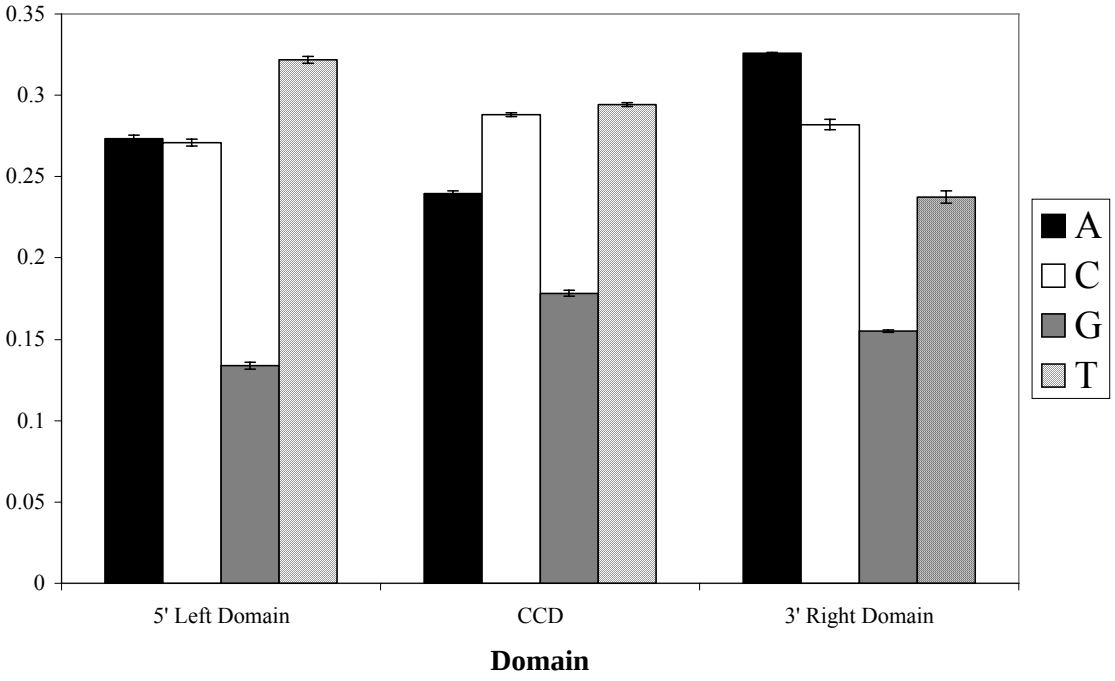



**Figure 3.2** Average base frequencies for each domain of the dugong control region (+/- standard error).

## ETAS1

```
B61       ACCATCCTATGTATAATCGTGCATTACACTACTTA-CC-CCATGC-ATA---T-AAGCCA--GTACAGTAG
SW1       .........................................G..........T..........
LEM1      .........................................G..........T..........
MD56      ............................................................
D3        ............................................................
T677      .......C....................................................G....
GB2       ............................................................
GB1       ............................................................G....
Cat       ..-..A........-...........A-T.G..AGT........A......T......AT..........
Dolphin   ..-..G........T..T.......CATT.-A..T....--..A.G...AGT.A.....C.....--T..A
Rabbit    ..-..A......T...........-.A.T.C..C.T..C.....A....A........-TA......T..-
Mouse     ..-..T-.......-.....A.....A.C.-A..TT......A..............-TA......T..A
Platyp    ..-..TA.......-..A..A.....A-T.G.A.GT...G....G......T.....AACCAA..----..A
B.Rhino   -.C-GGG.......-...........A.T.G-..TG.....................ATAT....TAC.T
Gibbon    ..-..-......C-T....A.....--..G.CAGT..............T..GTA.A-......TA..A
Hhog      ..AT.AAAT.A...-T.T-.A.TA..T.T.TA.GTAAT.T.TA..............AT.......T..A
```

## ETAS2

```
B61       CTCTTGATCTTGCATA--GTACATTCAACCCTTTGT----CGTACATAGCACATCTCTGAGATAGTTCTCGTCAACACGC
SW1       ........................T.....................................................
LEM1      ........................T.....................................................
MD56      ..............................A.............................C..T..........
D3        ............................T.....A.............................C..T..........
T677      T.............................A.....................................................
GB1       T.............................A.....................................................
GB2       ..............................A.....................................C..T..........
Cat       T.A.AT..A..A.....A-G....AAT.GT-GC.TAAT.....G...T-...C.TAA------TTC.A-G.A..G---T.
Dolphin   TCA..A..T..A......T.....GAT.TGTA.-AA.T...T......TT.T..--A------TCCC..AA-.....TTT
Rabbit    TC.ACT-.AA.A...-CAC-....A--.T..AACAAAAAAATTG..CC.-A....GAA------TATT...AC.....AAA
Mouse     TGG..C.-GG.-.....A--.-..A--.T.-A.CAA.....A..A..-CA.T..ATA------TAC--CA-.G.---ATA
Platyp    ACA..-..A..AA.A.TCC.-...ATC.TG-A..-A.T...C-.........AG.A-------..CC-----------...
B.Rhino   ..G.....T..A.....A-.....ATT.TT-A...A.T..........CGC...--C--------C--AA.......--AT.
Gibbon    .CA.CT.AAGG....G.A-.G..C..-.TT.A..CAC.....C.....CA.AC..C.------TACCACAC.......-.T.
Hhog      T.A..A..A..A............AAT.TT-A...A.T...T........G...-CC------TA.--.AA.A.---.TT
```

## CSB1 CSB2 CSB3

```
B61       TATTCAGTCAATGCTCGA-GGACATA   CAAACCCCCCTACCCCC      TGTCAAACCCCAAAAGCA
SW1       ........................    ...............        .................
LEM1      ........................    ...............        .................
MD56      ........................    ...............        .................
D3        ........................    ...............        .................
T677      ........................    ...............        .................
GB1       ........................    ...............        .................
GB2       ........................    ...............        .................
Cat       -............G..ACA.......   ...........TA....      ..C.........C..A..
Dolphin   -...T.A.T....G.TACA.......   T..........T.....
Rabbit    -....TT.T......T.TC.......   ..........CTA....      ..C............A.C
Mouse     A...-.T...-....T.TTA......   ...........-......      ..C.......A....A..
Platyp    ...AG.T.-......T..C.......   TTCC.......T.....
B.Rhino   -............G.TACA.......   ..........C......      ..C............A..
Gibbon    A...A.T...-....T.TT.......   ..........C..A...      ..C............A..
Hhog      A..-.TT.T....G.TACA.......   ...........TA....      ..A............C..
```

**Figure 3.3** Alignment of dugong sequences with identified regions from Sbisa *et al.* (1997). '.' Indicates similarity to the top sequence. '-' indicates a gap. B61, SW1, LEM1, MD56, D3, T677, GB1 and GB2 are dugong sequences. The remaining sequences are taken from Sbisa *et al.* (1997). Dolphin = Commerson dolphin (*Cephalorhynchus commersonii*), Rabbit = European rabbit (*Oryctolagus cuniculus*), Mouse = house mouse (*Mus musculus*), Platyp = platypus (*Ornithorhynchus anatinus*); B.Rhino = black rhinoceros (*Diceros bicornis*), Gibbon = *Hylobates lar*, Hhog = European hedgehog (*Erinaceus europaeus*). CSB3 does not occur in the Commerson dolphin or platypus control regions.

The microsatellite repeat region between CSB1 and CSB2 ranges from 246 bps long in dugongs T677 and D3 to 432 bps long in LEM1. Due to the difficulty of sequencing through such a long repeat in one direction, sequencing was done in both directions. Alignment of forward and reverse sequences was possible because some variation occurred in the repeat motifs present. The most common motif is CGCATA. Variants are shown in Table 3.5. The most complex of the motif arrangements is within dugong sequence GB2, which contains five out of the six repeat motifs identified in the dugong (Table 3.4).

**Table 3.5** Indication of the arrangement of motifs within the microsatellite repeat region (between CSB2 and CSB3) of the dugong and the paenungulates. The repeat motif and number of copies is given. Dugong sequences are B61, MD56, T677, SW1, D3, LEM1, GB and GB2. T. man = Florida manatee (*Trichechus manatus*), L. afr = African elephant (*Loxodonta africana*), E. max = Asian elephant (*Elephas maximus*), D. dor = Tree hyrax (*Dendrohyrax dorsalis*), P. cap1 = Rock hyrax (*Procavia capensis*) from GenBank, P. cap2 = Rock hyrax (*P. capensis*) sequenced this study. A = CGCATA; B = CA; C = TACA; D = CACGCA; E = CGCACA; F = TGTACA; G = CGTACA; H = CGCGTA; I = CGTATA; J = CGTATACA; K = CGCA; L = CTGCATA; M = GGCATA; N = TA; O = CTT; P = TGCATA.

| Species | Sequence | Motif arrangement in repeat region |
|---|---|---|
| Dugong | B61 | $A_{63}$ |
| | MD56 | $A_2B_3A_5B_3A_{60}$ |
| | T677 | $A_{41}$ |
| | SW1 | $A_{67}$ |
| | D3 | $A_{41}$ |
| | LEM1 | $A_3B_2C_1K_1A_4C_1A_7D_1A_{60}$ |
| | GB | $A_{10}D_1A_7D_1A_7D_1A_{17}D_1A_{19}$ |
| | GB2 | $A_2B_3A_4B_3A_2L_1A_1D_1A_5D_1A_{41}M_3A_2$ |
| Manatee | T. man | $A_1E_1F_1A_{64}$ |
| Elephant | L. afr | $G_1B_2N_1A_2G_3H_1B_2G_3H_1B_2G_6H_1B_2G_3A_{43}G_2I_1$ |
| | E. max | $G_1A_2P_1G_1B_1G_2E_1G_1B_2G_4B_2G_1E_1G_1E_2G_1A_2E_1K_1G_8H_1A_{27}I_2$ |
| Hyrax | D. dor | $J_{35}K_1$ |
| | P. cap1 | $J_{41}K_1$ |
| | P. cap2 | $J_{34}K_1$ |

### *3.3.3 Secondary structures present in the dugong control region*

Regions identified as ETASs by sequence alignment with previously published ETAS blocks (Sbisa *et al.*, 1997; Figure 3.3), did not form any strong secondary structures in the dugong. Similarly, the ETASs identified by Sbisa *et al.* (1997) also failed to form any strong secondary structures (Figures 3.4 and 3.5) when analyses in the same way as the dugong sequences. However, a folding of the entire 5' left domain did indicate, that

regions surrounding and including the identified ETAS 1 and 2 can form stable stems in the dugong (Figure 3.6). Four out of the 6 base pairs in each dugong microsatellite repeat can be involved in formation of a stem. (Figure 3.7). A similar arrangement is found in all paenungulate repeats. Similarly, the conserved sequence blocks themselves did not form secondary structures in the optimal folding (Figure 3.8), although CSB 2 and CSB 3 were suggested Sbisa *et al.* (1997) to be part of a complex stem and loop arrangement.

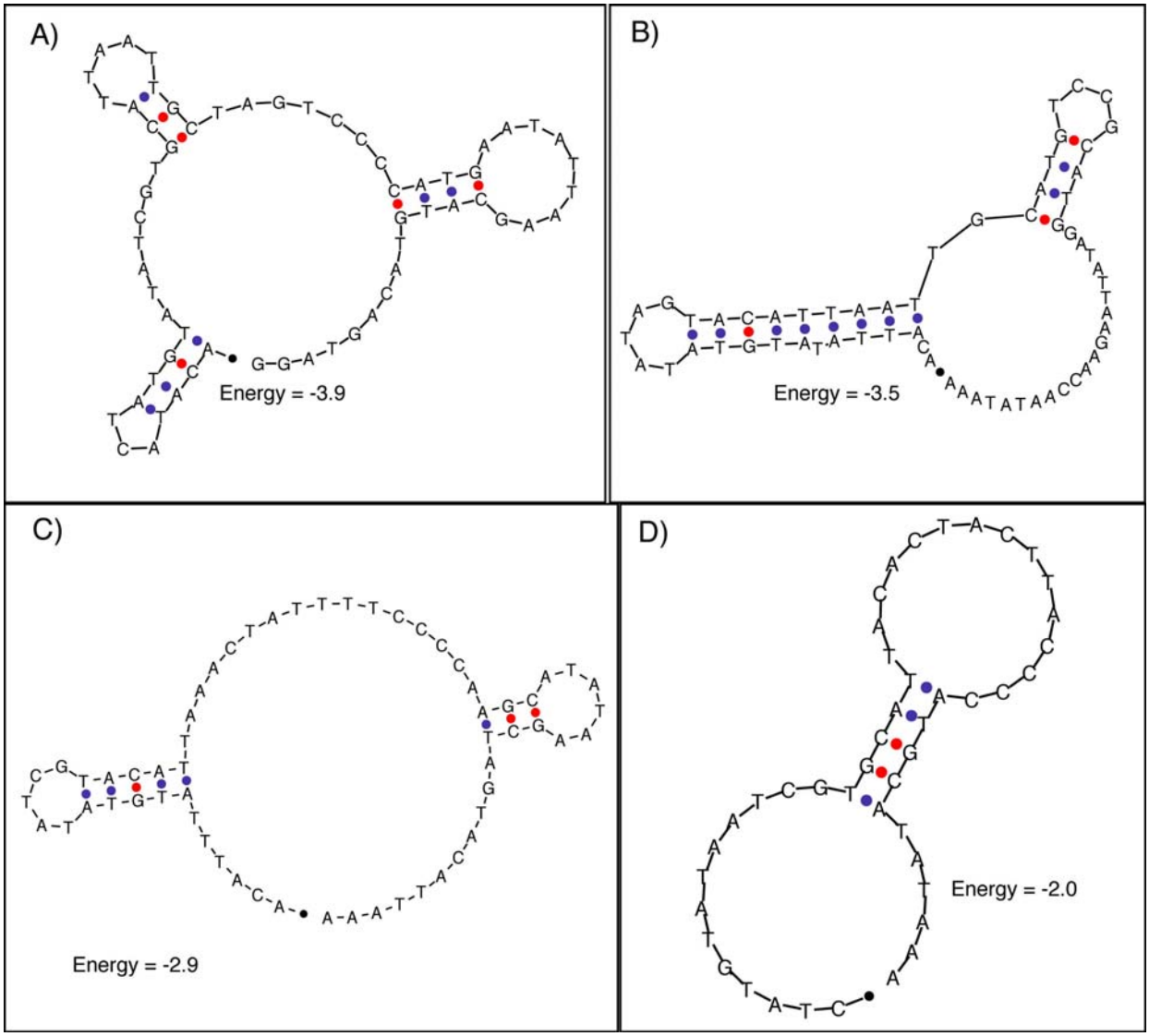



**Figure 3.4** Folding of the ETAS 1 region from selected mammals. Sequence data taken from Sbisa *et al.* (1997). Energy levels given in kcal/mole. A) Domestic cat (*Felis catus*), B) Platypus (*Ornithorhynchus anatinus*), C) House mouse (*Mus musculus*), D) Dugong (identified in this study). The black dots mark the 5' end of the sequences.

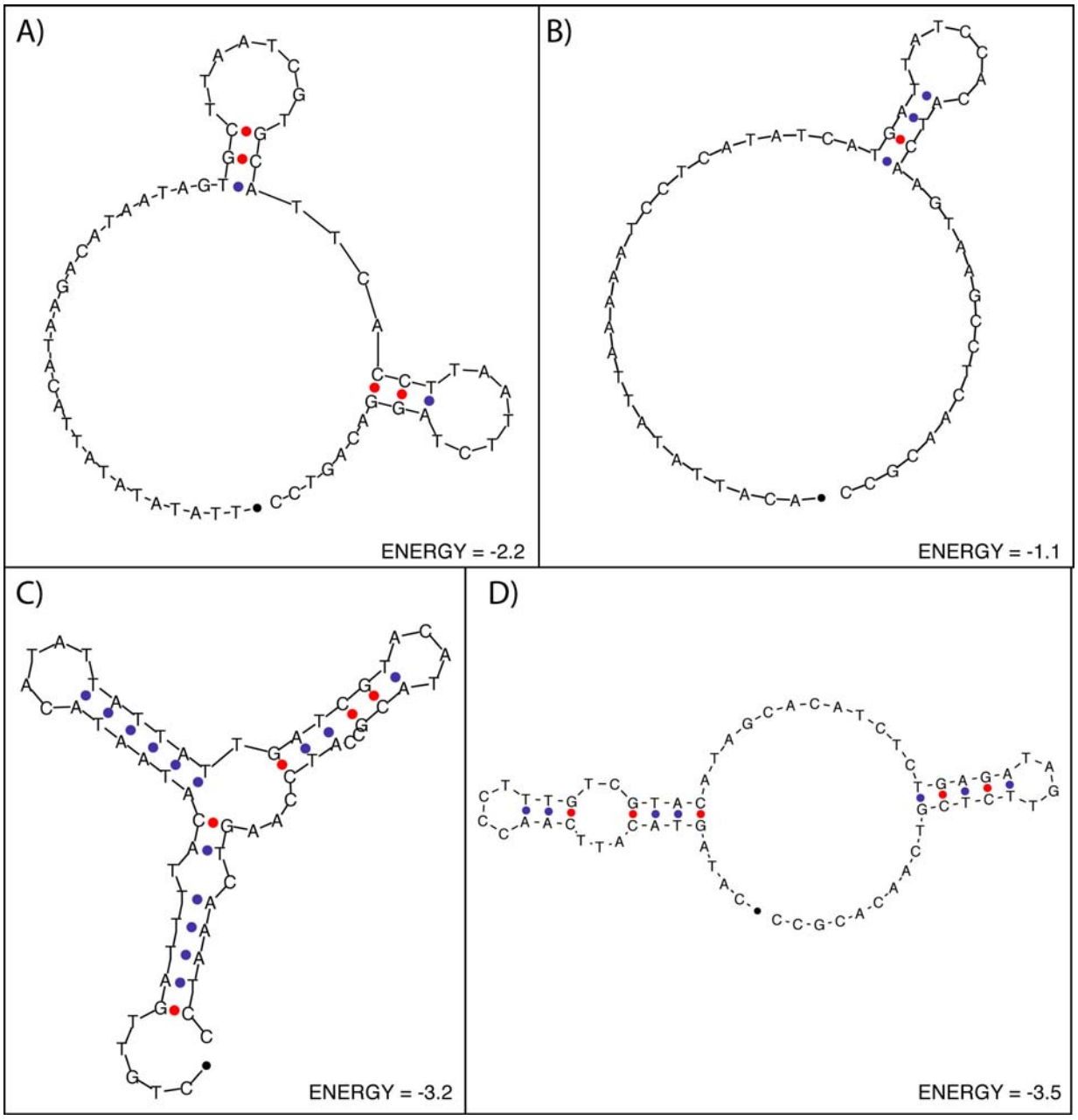



**Figure 3.5** Folding of the ETAS 2 region from selected mammals. Sequence data taken from Sbisa *et al.* (1997). Energy levels given in kcal/mole. A) Domestic cat (*Felis catus*), B) Platypus (*Ornithorhynchus anatinus*), C) Black rhinoceros (*Diceros bicornis*), D) Dugong (identified in this study). The black dots mark the 5' end of the sequences

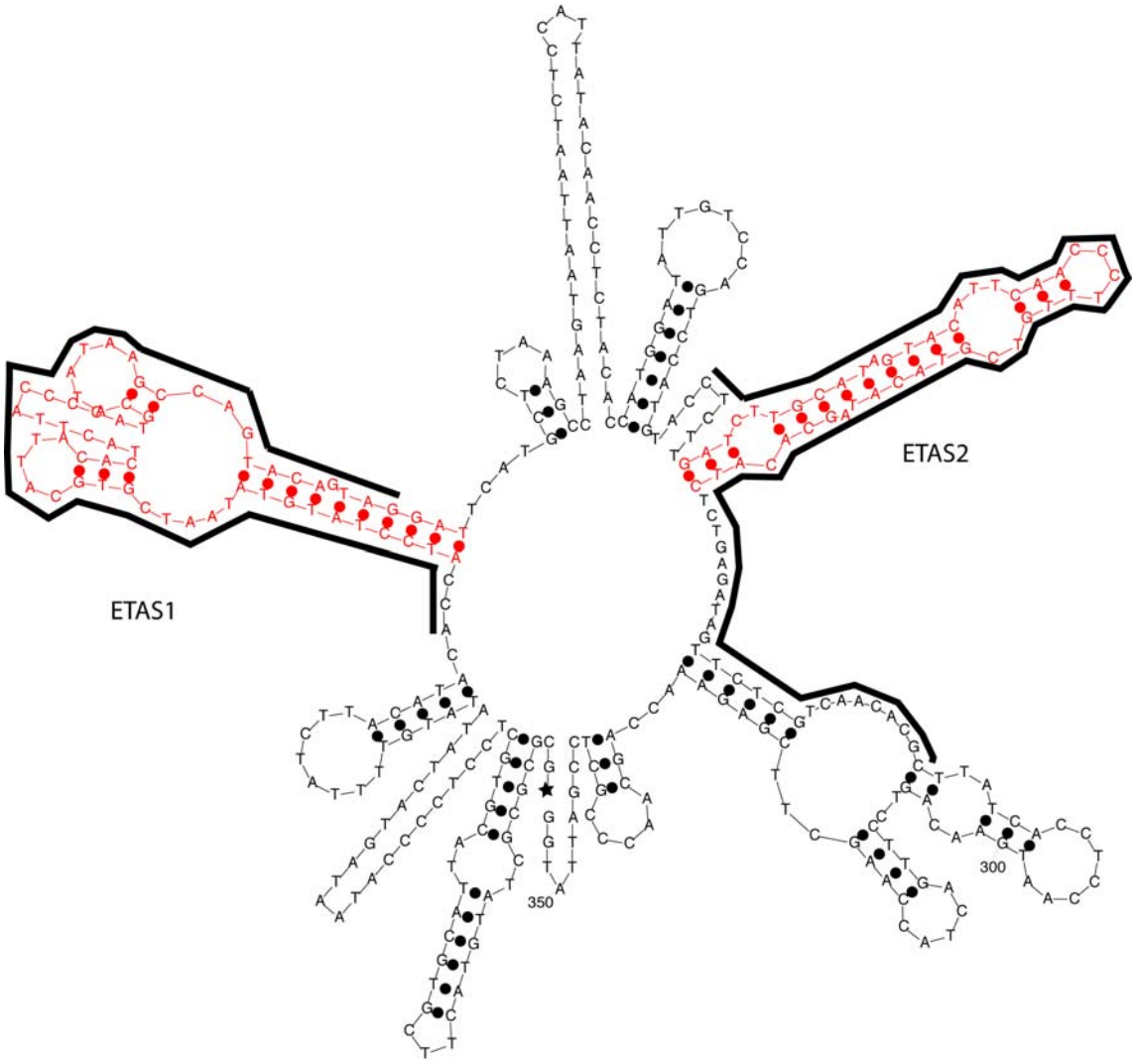



**Figure 3.6** Diagram showing the optimal secondary structure produced by the 5' left domain of the dugong control region (free energy of –25.0 kcal/mole). The dots indicate bonded base pairs. The ETAS 1 and 2 domains identified by sequence alignment are indicated with bold lines. These are the regions shown folded in Figures 3.4 and 3.5. The possible placement of the ETAS 1 and 2 based the ability to form stable stems are indicated in red. The star marks the 5' end of the sequence.

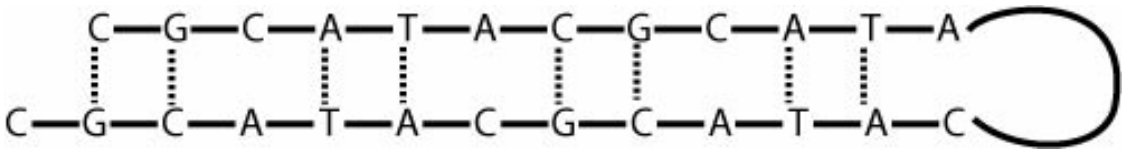



**Figure 3.7** Schematic showing the secondary structure of the common microsatellite repeat in the dugong. Bondings between bases are indicated with the dotted lines.

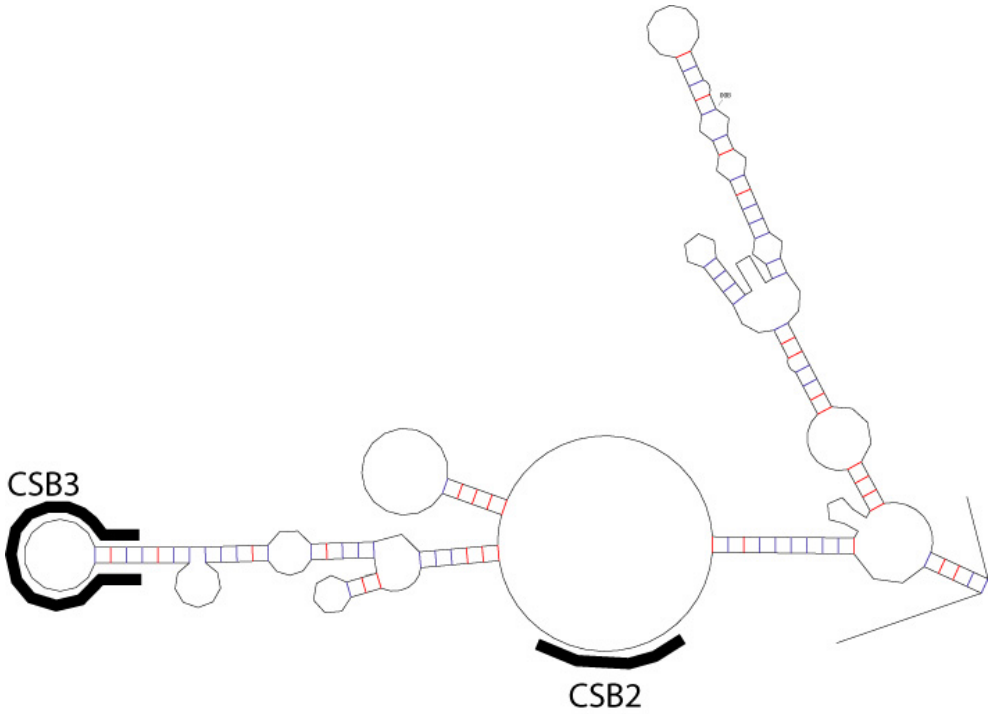



**Figure 3.8** Diagram showing the complex stem and loop structure containing the CSB 2 and 3 found in the dugong (free energy –46.5 kcal/mole). The CSBs are indicated with bold lines.

### *3.3.4 The variability in the dugong control regions*

The pairwise differences between completely sequenced dugong control regions ranged from 0.39 to 2.93%, with the greatest difference being between dugongs LEM1 (from the Philippines) and GB2 (unknown origin) (Table 3.6). The most variable domain in dugongs is the 5' left domain as indicated by the number of variable sites (section 3.3.2) and a pairwise difference up to 5.98% between LEM1 (Philippines) and T677 (Torres Strait) (Table 3.6). This variation is almost three times greater than that in the CCD and double the amount in the 3' right domain. Within the CSBs and ETASs the most variable section is ETAS 2 with a pairwise difference ranging from 0 to 6.76% followed closely by ETAS 1 with a range from 0 to 6.45% (Table 3.6). The CSBs were not variable within dugongs, which is not unexpected because of the conserved nature and the short length of each block.

**Table 3.6** Range of pairwise differences in percent between dugong control regions. The microsatellite region was included within the 3' right domain for these analyses.

| Region | Minimum (%) | Maximum (%) |
|---|---|---|
| Entire Control Region (1478 bp) | 0.39 | 2.93 |
| 5' Left Domain (350 bp) | 0.00 | 5.98 |
| ETAS 1 (62 bp) | 0.00 | 6.45 |
| ETAS2 (74 bp) | 0.00 | 6.76 |
| Central Conserved Domain (207 bp) | 0.00 | 1.93 |
| 3' Right Domain (920 bp) | 0.00 | 2.81 |

The control region of dugongs has 'little saturation' with an index of substitution saturation of 0.4130 (Iss.c = 0.8124; Table 3.7). This conclusion is also supported by the transition: transversion ratio of 15:3.

**Table 3.7** Results of test for saturation substitution, for the dugong and the Paenungulata overall. The interpretation of the index of substitution saturation (Iss) and critical value (Iss.c) is given below. The sequences included in each analysis and the dataset analysed are also identified. Probability values are conducted on a one-tailed test assuming a symmetrical tree. Sequence alignments are provided in Appendix 3.

| Sequences | Data set | Iss | Iss.c | Probability |
|---|---|---|---|---|
| Dugongs | Complete Control Region | 0.41 | 0.81 | 0.00 |
| Sirenia | Complete Control Region | 0.78 | 0.80 | 0.19 |
| Paenungulata | Alignment one | 0.95 | 0.79 | 0.00 |
| Paenungulata minus Hyracoidea | Alignment one | 0.80 | 0.80 | 0.43 |
| Paenungulata | Alignment two | 0.68 | 0.76 | 0.00 |

Interpretation of results (after Xia *et al.* 2003)

| | **Significant Difference** | |
|---|---|---|
| | Yes | No |
| **Iss < Iss.c** | Little saturation | Substantial saturation |
| **Iss > Iss.c** | Useless sequence | Very poor for phylogenetics |

### *3.3.5 Phylogeny and inter-specific similarity of the control region among the Paenungulata*

The alignment of all available paenungulate sequences showed a surprising similarity, especially between the sirenians and proboscideans (Appendix 3). The identified dugong structural features (Figure 3.3) were aligned with sequences from remaining paenungulates in order to identify the structural features in these sequences and to verify that these regions were indeed conserved. These include the three domains of the control region, ETAS 1 and ETAS2, and the CSB1, 2 and 3 (Figure 3.9). The tandem microsatellite repeat is also present in the other paenungulates, however the repeat in the rock hyrax and tree hyrax is CGTATACA, compared with the proboscidean and sirenian repeat of CGCATA (Table 3.5). The elephants also have a complex arrangement of repeat motifs, compared with the hyrax (Table 3.5).

## ETAS1

```
B61      TCCTATGTATAATCGTGCATTACACTACTTACCCCATGCATATAAGCCAGTACAGTAG
SW1      ....................................G.......T..........
LEM1     ....................................G.......T..........
MD56     ............................................................
D3       ............................................................
T677     ...C........................................................G....
GB       ............................................................G....
T. man   ................A...........A..........T.......A.........T
T. inu   ................A..........TA..........T.......AG....G...C
L. afr   .A.................C...T..T...........T.......A.....T..TT
E. max   .A.C...............C...T..T...........T.......A.....T..TT
P. cap1  C.........-........-.ACTG.....................A......-..-
D. dor   C.........-........-.A.T..TC.G..............T......-..-
```

## ETAS2

```
B61      CTCTTGATCTTGCATAGTACATTCAACC-CTTTGTCGTACATAGCACATCTCT-GAGA-TAGTTCTCGTCAACACGC
SW1      ......................T.....................................................
LEM1     ......................T.....................................................
MD56     ..............................A.............................C..T..........
D3       ........................T.....A.............................C..T..........
T677     T.............................A.....................................
GB       T.............................A.....................................
T. man   TC......A........C....CATG.TC...AA..............TA..T..A...CA.C..........T..
T. inu   TC......A........C....CAT...T...AA....C...........A..T..A...CA...........T..
L. afr   AC.A.AG....A.....C.....A..G.T...G................A.......AA-TC...A....C..T..
E. max   AC.A.AG....A.....C.....A..G.T...GA...........G...TA.......AA-T....A....T..T..
P. cap1  ------..-..AA..A-.A...AAAT.AA...GAA..AAG..C......CAA.....C.AA.A...AA.C.......G
D. dor   ------..-..AA.CA-CA...AAATCAG.A.GAA.GA-CT...AG...CAA.....CTAA.AC..CA.A........
```

## CSB1

```
D. dug   AATGCTCG-AGGACATAGAA
T. man   ..............G..A..
L. afr   ........G........A..
D. dor   ..G..K........NG..--
P. cap1  ......T.A........A--
P. cap2  ........-.....C..A.-
```

## CSB2

```
D. dug   CAAACCCCCC--TACCCCCC--TTAAT--T
T. man   .........A........A...........
L. afr   ...........T.............G....
D. dor   .....A....CC......T.TTGG.C.TT.
P. cap1  ..........CC.........CCG..C.CT.
P. cap2  ..........CC.........CCG..C.CT.
```

## CSB3

```
D. dug   CTTGTCAAACCCCAAAAGCA
T. man   ....................
L. afr   .C......-...........
D. dor   A...G...............
P. cap1  ....................
P. cap2  ....................
```

**Figure 3.9** Alignment of regions of interest in the paenungulate control region - conserved sequence blocks (CSB) and extended termination associated sequences (ETAS). B61, SW1, LEM1, MD56, D3, T677, and GB are all dugong sequences. D. dug = dugong (*Dugong dugon*) sequence where all individuals were the same. T. man = Florida manatee (*Trichechus manatus*), L. afr = African elephant (*Loxodonta africana*), E. max = Asian elephant (*Elephas maximus*), D. dor = Tree hyrax (*Dendrohyrax dorsalis*), P. cap1 = Rock hyrax (*Procavia capensis*) from GenBank, P. cap2 = Rock hyrax (*P. capensis*) sequenced this study. A '.' indicates similarity with the top sequence and '-' indicates a deletion.

Tests on alignment one of the Paenungulata suggested saturation (Table 3.7). The use of the paenungulate alignment two was supported by saturation substitution tests indicating that alignment two sequences appear to be unsaturated (Table 3.7). Alignment two was then used for all further phylogenetic analyses.

The hyracoideans appear to be the most divergent paenungulate order with percentage differences between Hyracoidea and Sirenia, and between the Proboscidea and Hyracoidea, ranging from 23.1% to almost double the differences between the dugongs and manatees (Table 3.8a). As expected, the most similar domain across all the paenungulates is the central conserved domain (Table 3.8b). This domain contrasts with the 5' domain, which is highly variable (Table 3.8b). As also seen in comparisons between dugongs, the ETAS 2 has a greater difference among the paenungulates than ETAS 1, with a range of 17.3 to 57.0% compared with 8.6% to 20.4% differences among the ETAS1 (Table 3.8c and Figure 3.9). Although the CSBs are short, variation was identified among the paenungulates with the CSB2 having the most variation (Table 3.8d). The tree hyrax (*D. dorsalis*) contains only a partial CSB3 (Figure 3.9).

Phylogenetic trees consistently grouped the sequences into the established orders. In total, six equally most-parsimonious trees were produced from a heuristic search. These trees differed only in the placement of terminal branches within the Sirenia. Despite the divergence of the hyracoidean mentioned above, all phylogenetic analyses suggested a closer relationship between the Hyracoidea and Sirenia than between Sirenia and Proboscidea (Figure 3.10). The parsimony bootstrap analyses clustered the Hyracoidea with the Sirenia 61% of the time (Figure 3.10). Interestingly the outgroup taxon, the cape golden mole, appears as a sister taxon to the hyracoideans (Figure 3.10). Phylogenetic analyses conducted on the central conserved domain alone, all showed a similar relationship between the paenungulate orders and outgroups (trees not shown). Analyses conducted without the cape golden mole still produced the same relationship between paenungulate orders (trees not shown).

**Table 3.8** Percentage pairwise differences of the control region between the paenungulates using the uncorrected 'p' distance measure. All analyses were conducted on the alignment two. A) Percent differences of the control region sequences between the paenungulates. B) Percent differences of the three domains (5' left domain, Central conserved domain and 3' right domain) among the paenungulates. C) Percent differences of the ETAS 1 and 2 regions in the paenungulates. D) Percent differences of the CSBs among the paenungulates.

**A) Control Region Overall (857 bp)**

| Orders/Families | Minimum distance | Maximum distance |
|---|---|---|
| Dugongidae - Trichechidae | 14.4 | 20.2 |
| Sirenia - Proboscidea | 21.8 | 22.6 |
| Sirenia - Hyracoidea | 22.2 | 34.1 |
| Hyracoidea - Proboscidea | 23.1 | 32.7 |

**B) Three Domains Compared**

| Orders/Families | 5' Left Domain (283 bp) | | CCD Domain (209 bp) | | 3' Right Domain (365 bp) | |
|---|---|---|---|---|---|---|
| | Min | Max | Min | Max | Min | Max |
| Dugongidae - Trichechidae | 20.6 | 23.1 | 2.8 | 4.3 | 12.1 | 12.5 |
| Sirenia - Proboscidea | 29.2 | 33.2 | 4.8 | 7.7 | 20.3 | 21.0 |
| Sirenia - Hyracoidea | 34.4 | 39.1 | 5.3 | 9.1 | 27.5 | 28.3 |
| Hyracoidea - Proboscidea | 36.9 | 37.9 | 5.2 | 8.1 | 27.9 | 30.1 |

**C) ETAS1 and ETAS2**

| Orders/Families | ETAS1 (58 bp) | | ETAS2 (77 bp) | |
|---|---|---|---|---|
| | Min | Max | Min | Max |
| Dugongidae - Trichechidae | 8.6 | 13.8 | 17.3 | 24.4 |
| Sirenia - Proboscidea | 13.7 | 17.2 | 22.8 | 30.6 |
| Sirenia - Hyracoidea | 10.6 | 20.4 | 43.6 | 57.0 |
| Hyracoidea - Proboscidea | 16.3 | 18.2 | 40.7 | 49.3 |

**D) Conserved Sequence Blocks**

| Orders/Families | CSB1 (20 bp) | | CSB2 (30 bp) | | CSB3 (20 bp) | |
|---|---|---|---|---|---|---|
| | Min | Max | Min | Max | Min | Max |
| Dugongidae - Trichechidae | 4.0 | N/A | 11.1 | N/A | 0.0 | 0.0 |
| Sirenia - Proboscidea | 0.0 | 8.3 | 0.0 | 10.5 | 0.0 | 0.0 |
| Sirenia - Hyracoidea | 3.8 | 16.8 | 5.6 | 25.9 | 0.0 | 5.5 |
| Hyracoidea - Proboscidea | 3.8 | 20.6 | 5.4 | 15.4 | 0.0 | 5.5 |

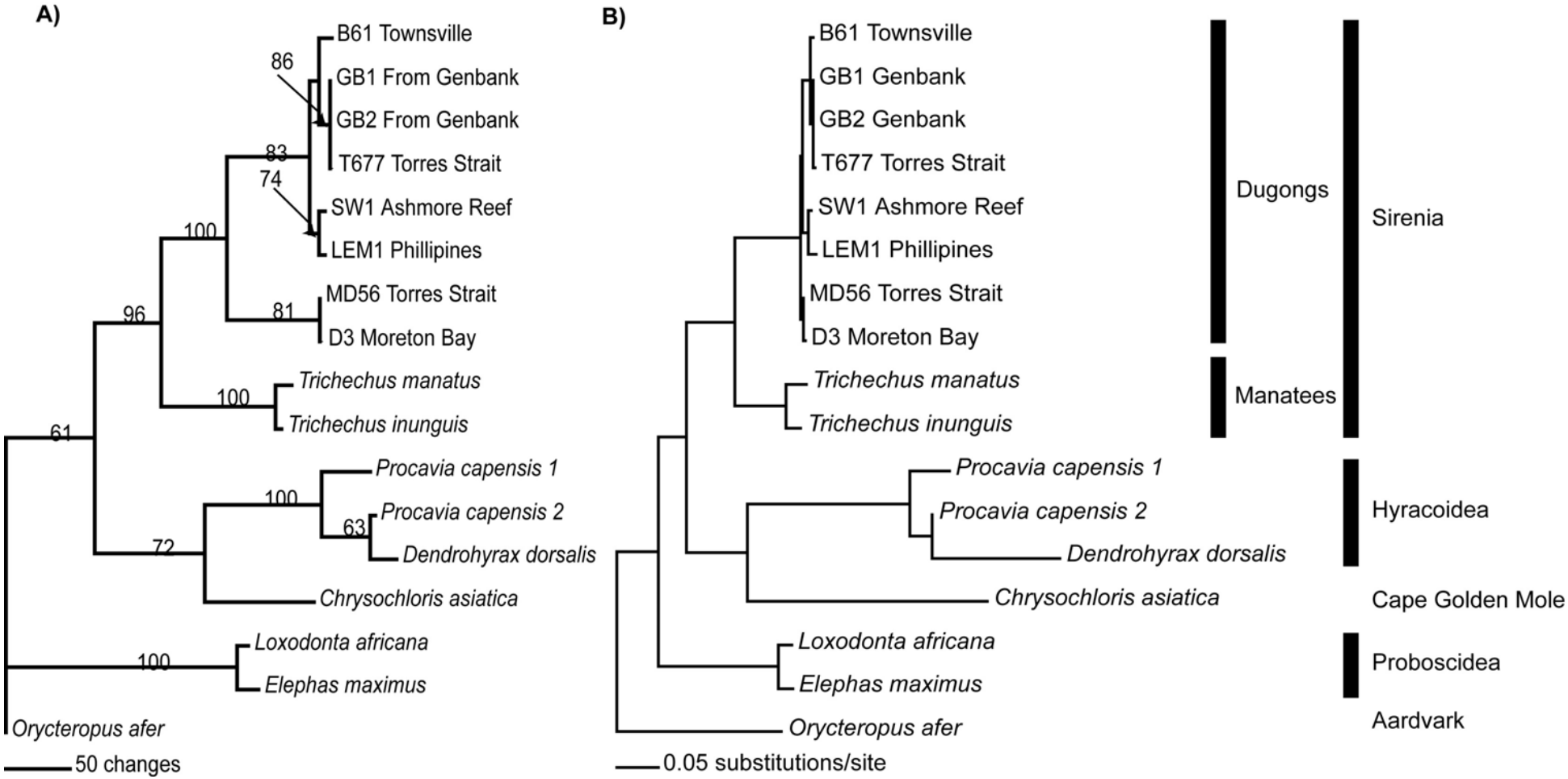



**Figure 3.10** Phylogenetic analyses of the paenungulate orders based on the mtDNA control region (alignment two). *Orycteropus afer* (aardvark) and *Chrysochloris asiatica* (cape golden mole) were used as outgroup taxa. Aardvark, Cape Golden Mole, all proboscidean sequences, *Procavia capensis 1, Trichechus inunguis*, GB1 and GB2 were obtained from GenBank (See Table 3.1 for accession numbers). Dugong sequences D3 Moreton Bay and T677 Torres Strait were taken from Tikel (1997), all other samples were sequenced in this study. The relevant clades are indicated. A) Parsimony consensus tree incorporating branch lengths, with parsimony bootstrap values indicated on branches (500 replicates). B) Maximum likelihood tree.

## 3.4 Discussion

### *3.4.1 Most variation of the mitochondrial genome is in the control region*

The comparison of the two published mitochondrial genomes of dugongs indicated that the control region contains a high level of variation. Analyses also indicated that the two genomes were from individuals that represent the two mtDNA lineages of dugongs within Australia (data not shown this chapter – see Chapter 4), this result means that the variation present is representative of that within Australian dugongs. The control region was chosen for the phylogeographic study (Chapter 4). I evaluated its properties including its structure and variation among dugongs. As part of this investigation I wanted to determine if the control region would produce a credible phylogeny. To do this, a study on the variation of the Paenungulata (*i.e*. the dugong's closest relatives) was carried out.

### *3.4.2 Similarities of the dugong control region to that of other mammals*

The dugong control region is typically mammalian in its length, base composition and structural arrangement of identifiable motifs. However, the base composition in the 5' left domain differs from the typical mammal control region described by Sbisa *et al.* (1997). Dugongs have a high T content followed by A > C > G in the 5' left domain (Figure 3.2). Compared with A > T > C > G as described in Sbisa *et al.* (1997) as typical for mammals in both the 5' and 3' domains. If the microsatellite repeat (section 3.3.2; Table 3.5) is included in the analysis of the 3' right domain, the proportion of C becomes greater (A > C > T > G). The higher C content in the 3' right domain is a result of the number of Cs in the microsatellite repeat. Overall, as in other mammals, the control region of dugongs is G-poor.

Sbisa *et al.* (1997) suggested that the ETAS 1 and ETAS 2 regions appear to have the ability to form secondary or tertiary structures which contain recognition features responsible for the termination of replication. This result was used to explain the presence of these regions in all mammal control regions studied by Sbisa *et al.* (1997). Sbisa *et al.* (1997) indicated that strong secondary structuring occurred in these regions although secondary structure diagrams were not presented. The analyses I conducted on the ETAS regions identified by Sbisa *et al.* (1997) did not produce any strong

secondary structures (Figures 3.4 and 3.5). All structures were highly variable between mammal species and consisted of stems as short as 3 base pairs (Figures 3.4 and 3.5). In contrast with the statements by Sbisa *et al.* (1997), the ETAS regions identified in the dugong do not correspond to whole stems when the entire 5' left domain is folded (Figure 3.6). It is important to note that identification of these ETAS blocks based on alignment of published sequences may not indicate the true regulatory regions, especially in the highly variable 5' left domain. If these ETAS blocks are truly regulatory regions then a combination of sequence alignment and secondary structure analysis should allow for their proper identification. With this in mind the potentially functional ETAS regions in dugongs were indicated in red on Figure 3.6.

The presence of CSB1 within the dugong is not unusual, as this section is thought to be important for primer generation during the replication of the mtDNA (Sbisa *et al.* 1997). The presence of the origin of the heavy strand replication (OH) near or in CSB1 indicates the importance of this region in replication (Sbisa *et al.*, 1997). However, the variability seen within CSB1 among the Paenungulata contradicts the importance of this CSB compared with CSB2 and CSB3 where differences were far fewer (Table 3.8). CSB2 and CSB3 are not always present in mammals, although they are both present in the dugong and the other paenungulates, except that only a partial CSB3 occurs in the tree hyrax (Figure 3.9). This lack of the CSB2 and CSB3 in other mammals indicates that they are not as vital to the replication of the mtDNA. It was suggested by Sbisa *et al.* (1997) however, that the spacing of these conserved blocks and their ability to produce secondary structures may make them responsible for the packaging of the d-loop region for replication. Analyses conducted on the dugong 3' right domain and CSBs do not appear to indicate highly stable secondary structures, although CSB2 and CSB3 are part of a complex stem and loop arrangement (Figure 3.8). At present the exact functions of the conserved regions within the dugong control region cannot be established. The importance of CSB1 is questionable, given the higher degree of similarity between the paenungulates found for CSB2 and 3. However, the presence of these regions in all mammalian control regions analysed thus far indicates that they do serve some purpose, most likely aiding in the binding of important proteins responsible for replication of the mtDNA.

The presence of microsatellite-like repeats appears to be a common feature of vertebrate mitochondrial control regions. These repeats always occur in the same position between the CSB1 and CSB2 [identified in rabbit, rhinoceros, horse, donkey, shrew, hedgehog, elephant seal, harbour seal, grey seal, cat and opossum - Sbisa *et al.* 1997; also in the aardvark, cape golden mole and all paenungulates (this study)]. Sbisa *et al.* (1997) noted that such repeats are formed of motifs that can fold into self-complementary secondary structures whose stability increases with the number of repeats. This self-complementary folding does appear to occur in the repeats identified within Sirenia (Figure 3.7): four out of six bases in each repeat are able to participate in stem formation. However, the complex arrangement of motifs within the microsatellite repeat (Table 3.5) may aid in forming stronger secondary structures.

The presence and similarity of these microsatellite-like tandem repeats in all paenungulates studied suggests these repeats may originate from a similar motif in a common ancestor, with descendant species undergoing additional deletion and insertion events. The insertions and deletions may produce variable copy numbers and repeat types that may differ only by one or two bases. However, the CGCATA repeat present in the dugong has also been identified in carnivores (Hoelzel *et al.*, 1994), which suggests convergent evolution or homoplasy.

Short repeats (such as the microsatellite repeat in the paenungulates) within the control region are identified as the main cause of heteroplasmy (the presence of two different copies of a gene in the same individual) in the mtDNA of carnivores (Hoelzel *et al.*, 1994). This heteroplasmy is a result of differences in repeat copy numbers (Hoelzel *et al.*, 1994). The repeats positioned between CSB1 and CSB2 show the highest level of heteroplasmy (Hoelzel *et al.*, 1994). Heteroplasmy was not identified in the dugong; however the entire repetitive region could not be sequenced in a single pass because of its length. The presence of multiple bands for this section in the tree hyrax, rock hyrax and manatee (requiring cloning of the PCR products before sequencing could be done - see section 3.2.2) appeared to result from non-specific priming. However they may instead be a result of heteroplasmy.

### *3.4.3 Paenungulate similarity and divergence estimates*

For a group of organisms thought to have diverged 65 MYA (Lavergne *et al.*, 1996) the control regions of the Paenungulata are surprisingly similar to each other, especially between Proboscidea and Sirenia (Table 3.8). The sequence similarity is unexpected given the rapidity of evolution and substitutions thought/known to occur (Gillham, 1994) within the mitochondrial control region. Because of this, the control region is widely considered to be the most rapidly diverging region of the mitochondrial DNA molecule, as appears to be within the dugong (Table 3.3). In contrast, Brown *et al.* (1986) found the average base substitution rate in the control region to be similar to that of the coding regions of the mtDNA. Brown *et al.* (1986) indicated that the proportion of base substitution within the control region between mouse and rat is approximately 15% which is similar to that found (14% to 27%) for a number of protein coding genes. Similarly, Pereira *et al.* (2004) showed that in cracid birds the control region evolves at a slower rate than protein coding genes, which is consistent with basal avian lineages having slower rates of evolution compared to more recent avian lineages. The Paenungulates are considered to be one of the more basal mammalian lineages and it is therefore possible that they are evolving at a slower rate than more recent mammalian lineages. However, a comparison of the divergence among the paenungulates within protein coding genes may confirm this slow rate of evolution. Contrasting this, a study by Parr (2000) on the position of Sirenia within the paenungulates found significantly different rates of evolution between the Proboscidea and Sirenia. She therefore did not carry out any comparisons of the mtDNA control regions between orders. This lack makes it difficult to compare her results with those obtained here.

One explanation of the similarity among paenungulates is that the control region sequences have reached saturation. However saturation is unlikely to explain similarities between sequences unless there are only very few sites which are free to vary – within the mtDNA control region this is presumably not the case. This result is supported by the substitution saturation tests conducted on alignment two of paenungulate control region, which suggest that saturation has not been reached yet (Table 3.7). This conclusion contrasts with the results of Parr (2000) who assumed saturation of nucleotide changes as a result of the low transition/transversion ratio of 4:5 for Sirenia. The most probable reason for this contrasting result is that Parr (2000)

examined a segment that included part of the cytochrome *b* gene and a short section of the control region, which contains a high proportion of variable sites.

### *3.4.4 Phylogenetic relationship among Paenungulata*

The sequence similarity between Sirenia and Proboscidea (Table 3.8) contrasts the positioning of Sirenia and Hyracoidea as sister taxa in the phylogenetic trees produced (Figure 3.10). It is therefore apparent that the differences between Sirenia and Proboscidea are more phylogenetically important than differences between Sirenia and Hyracoidea. Although there is a low level of resolution among the paenungulate orders in as indicated by the low bootstrap values (Figure 3.10) the phylogenetic relationships suggested by this study is supported by three published molecular studies (Porter *et al.*, 1996; Ozawa *et al.*, 1997; Nishihara *et al.*, 2005). However this study contrasts with other molecular studies that have included sequences of all representative paenungulate orders (Table 3.9) (Stanhope *et al.*, 1996; Parr, 2000). The latter studies suggest that the Hyracoidea has a closer relationship to Proboscidea than to the Sirenia (Table 3.9). This is contrary to the Tethytheria hypothesis, which has been supported by molecular studies (Kleinschmidt *et al.*, 1986; Lavergne *et al.*, 1996; Nishihara *et al.*, 2005), that suggests a grouping of Proboscidea and Sirenia (Table 3.9).

**Table 3.9** Summary of molecular studies on the relationships among Paenungulata. Results from this study are indicated in bold.

| **Relationship supported among Paenungulata** | **References** | **Molecular marker** |
|---|---|---|
| Tethytheria (Sirenia/Proboscidea) | Kleinschmidt *et al.* (1986) | Hemoglobin |
| | Lavergne *et al.* (1996) | 12s rRNA |
| Hyracoidea/Proboscidea | Parr (2000) | 3' end of cytochrome b gene, tRNAs threonine and proline. |
| | Stanhope *et al.* (1996) | Interphotoreceptor Retinoid Binding Protein |
| | Nishihara *et al.* (2005) | Nuclear genes |
| Sirenia/Hyracoidea | Porter *et al.* (1996) | von Willebrand Factor gene |
| | Ozawa *et al.* (1997) | cytochrome *b* gene |
| | Nishihara *et al.* (2005) | SINE inserts |
| | **This Study** | **Alignment two** |

It is clear from this and previous molecular studies that the Paenungulata is a monophyletic super-ordinal group. The exact relationships among the orders have not been clearly established. One reason for this is that the species divergence event that defined the three orders of the Paenungulata occurred over a relatively short evolutionary time period. Because of this rapid speciation, Nishihara *et al.* (2005) suggest that the tree produced from mtDNA studies is more representative of the species tree as a result of the faster coalescence time of mtDNA compared with nDNA. It is likely that with additional sequences from hyrax species and individuals, and additional loci (both non-coding and coding sequences) the relationships between the paenungulates will be further resolved. Suggestions for additional regions within the mitochondrial genome include ND5 (with approximately 23 base changes occurring in this gene between the two complete dugong genomes in GenBank), and cytochrome *b* (a useful and often-used gene which has a lower rate of variability hence making it useful for inter-specific comparisons).

The close relationship between hyracoideans and the outgroup taxon, the cape golden mole, suggests that the Paenungulata may not be a distinct superorder but a paraphyletic subset of the Afrotheria. Nishihara *et al.* (2005) also included the golden mole in their dataset and found that it appeared to be well situated within a clade with the Aardvark and tenrecs. This contrasting result emphasises the need for further analyses using additional taxa to understand the phylogenetic relationship between the Paenungulata and Afrotheria.

### *3.4.5 Choice of mitochondrial marker for phylogeographic studies in dugongs*

Analyses of the entire mitochondrial genome suggest the control region contains the highest number of polymorphic sites. It is therefore ideal to use this region for intra-specific phylogenetic studies. Closer examination of this region identified the most appropriate section of the control region for use in a phylogenetic study among dugongs as the 5' left domain, an area containing the greatest number of variable sites (Table 3.6). The difficulty in sequencing the 3’ domain due to the long microsatellite repeat makes this section less suitable for a phylogeographic study although this section contains a high amount of variability.

### *3.4.6 Importance to this study on dugongs and to future studies*

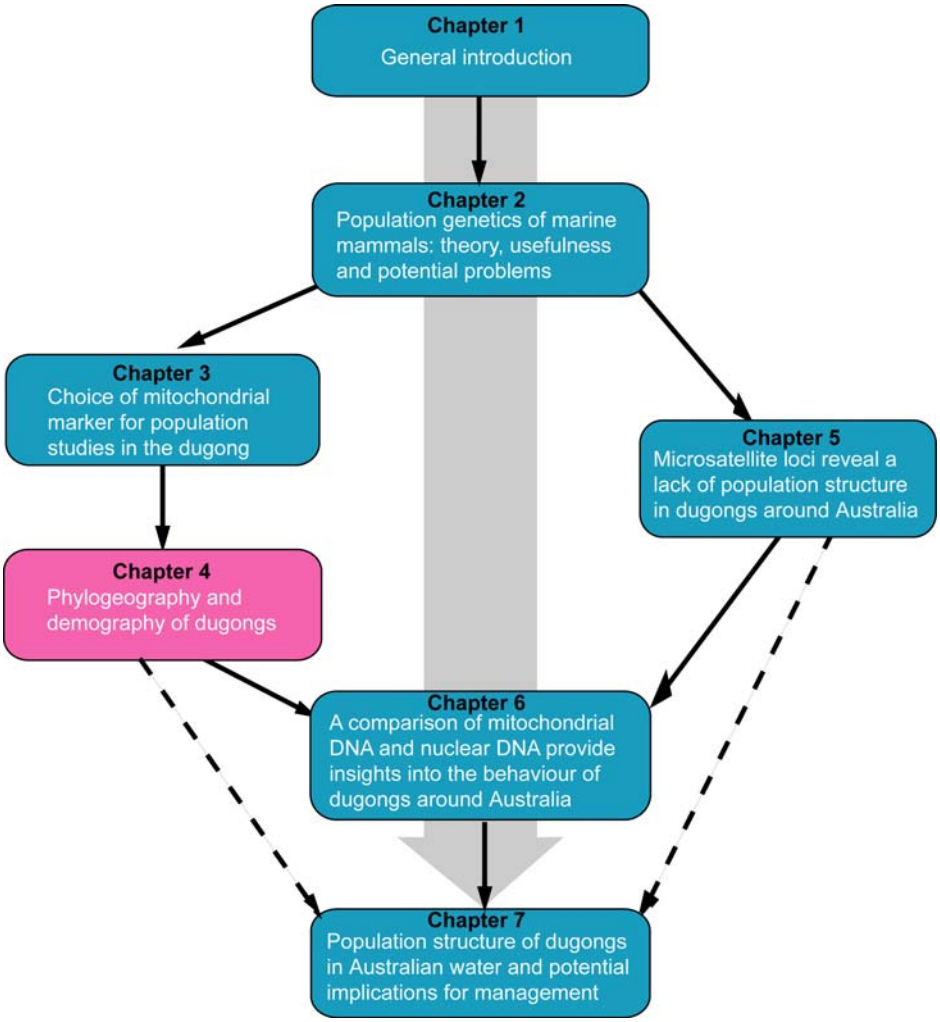
This chapter has extensively characterised the structural organization of the dugong mitochondrial control region using data from eight dugongs (including four newly sequenced individuals). This characterisation includes the analysis of base composition, identification of conserved regions, ability to form secondary structures and the sequence divergence within dugongs. This study is the first to include a full mitochondrial control region sequence from the Florida manatee and partial sequences from the tree hyrax. I also suggest the possibility that the control region may be evolving at a slower rate in paenungulates than in other mammals. Phylogenetic analyses of the Paenungulata suggest that the Sirenia is most closely related to the Hyracoidea, disagreeing with the Tethytheria hypothesis (Proboscidea + Sirenia). This study has also raised the possibility of the Paenungulata being a subset of the Afrotheria and not a distinct superorder.

This chapter has identified a number of additional areas of further study. These include the importance and applicability of secondary structures to the characterisation of the mitochondrial control regions in mammals, heteroplasmy within the microsatellite repeat of dugongs, and the usefulness of identification of conserved areas, such as the ETAS 1 and 2, solely by sequence alignment especially in regions known to be highly variable.

In this chapter, I assessed the usefulness of the control region as a molecular marker for population studies in the dugong. This region has the highest level of variation within the entire mitochondrial genome of dugongs (Table 3.3). I therefore described the structural organization of the dugong control region, and the amount of intra-specific variability occurring in this region and identified the 5' domain of the control region as the most variable. I also revealed that the control region can successfully produce reasonable phylogenies of the Paenungulata. The knowledge that this specific region appears to be slowly evolving in the superorder Paenungulata is important as it places any variability and divergence among dugongs in an evolutionary context. The application of this region in phylogeographic analyses has been conducted in chapter 4.

# Chapter 4: Phylogeography and demography of dugongs around Australia

*This chapter presents a phylogeographic study of dugong populations around Australia using the 5' end of the mtDNA control region. This study provides evidence of two maternal lineages of dugongs around Australia, which overlap in distribution in the Great Barrier Reef World Heritage Area and Torres Strait. A possible explanation of the pattern is the past subdivision of dugong populations due to the presence of a land bridge at times of low sea level such as during the last glacial maximum. The incomplete geographic overlap of the two lineages is unexpected and may be indicative of behavioural characteristics of this species not yet recognised, such as female dugongs returning to known seagrass habitats to give birth and raise young.*



## 4.1 Introduction

As discussed in chapter two, phylogeography is the area of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially at the intra-specific level (Avise, 1998; Avise, 2000). Phylogeography requires input from numerous disciplines including molecular and population genetics, ethology, demography, phylogeny, palaeontology, geology and historical geography (Avise, 2000). For dugongs there is a lack of data in many of these areas, which emphasises the importance of this study in providing basic information on this vulnerable species.

### *4.1.1 Dugong fossil record*

Modern dugongs have a tropical to subtropical distribution throughout the Indo-West Pacific region, similar to that of the tropical seagrasses on which they feed (see section 1.2). There is little fossil evidence available for the dugong or any other dugongids in this region, so our understanding of the palaeontological history of dugongids is based on knowledge obtained from the fossil-rich Caribbean. This record indicates that the Sirenia was once a speciose and diverse group, having undergone a major radiation during the Oligocene (Domning, 2001). The Dugongidae first appeared in the Mediterranean region in the fossil record of the middle to late Eocene and, based on numerous widespread fossil deposits, is considered the most diverse and successful family of the order Sirenia (Domning, 2001). The extant dugong (the only representative of the Dugongidae alive today) has a limited fossil record only represented by several fragments from the Pleistocene era from Papua New Guinea and Australia (Domning and Furusawa, 1995; Domning, 2001). These fragments include a vertebra, rib and skull from Papua New Guinea (de Vis, 1905), and dugong subfossils from Australia (Etheridge *et al.*, 1897; Etheridge, 1905). The most recent complete dugongid fossil was from the late Pliocene of Florida (Domning, 2001) and dates from the end of the period when dugongids occurred in the West Atlantic, a region now occupied solely by trichechids (Domning, 2001).

### *4.1.2 Dugong vagility*

Like most marine mammals, dugongs can move large distances. However there appears to be no distinct migration season in the tropics, and the direction and distance of movement varies between individual animals (Sheppard *et al.*, in press). At the low latitude extremes of their range, movements of dugongs are in response to cold water temperatures (Sheppard *et al.*, in press). Large-scale movements are not uncommon with 14 out of 72 dugongs that were satellite tagged moving distances greater than 100 km and 28 dugongs moving between 15 –100 km (Sheppard *et al.*, in press).

Most results from movement studies using satellite tracking have revealed the movement behaviour of dugongs within a bay. This scale may not be appropriate to gather an overall picture of the amount of movement and dispersal that dugongs are undertaking. Current evidence from aerial surveys of dugong populations in Torres Strait and the Great Barrier Reef World Heritage Area and Shark Bay, Western Australia indicate substantial changes in population numbers (thousands) over five year periods (Marsh *et al.,* 1997; Marsh and Lawler, 2001, 2002; Gales *et al.*, 2004; Marsh *et al.*, 2004). Given dugong life history, this change in numbers cannot be attributed to natural fluctuations in local populations (Marsh *et al.*, 1997; Marsh and Lawler, 2001, 2002; Gales *et al.*, 2004; Marsh *et al.*, 2004). It is therefore considered likely that large numbers of individuals to have moved between survey areas (Marsh *et al.*, 1997; Marsh and Lawler, 2001, 2002; Gales *et al.*, 2004; Marsh *et al.*, 2004). This evidence along with satellite tracking of individuals indicates that dugongs have high vagility.

### *4.1.3 Barriers to dugong movements*

In general we can infer that the dugong encounters very few barriers to movement and dispersal, other than wide and/or deep expanses of ocean (Martin and Reeves, 2002). However, fragmented resources and historical land bridges may have produced barriers in the past. Dugongs are dependent on tropical seagrasses as a food source (Lanyon *et al.*, 1989). These seagrass meadows are typically distributed patchily along coastlines and dugong distribution also tends to be discontinuous. Habitat fragmentation or presence of new barriers may result in reduced migration or gene flow between populations of a species (Frankham *et al.*, 2002). If prolonged, the lack of migration

would lead to increased genetic distance between populations and a loss of genetic diversity within smaller populations (Frankham *et al.*, 2002). However, with the dugong's ability to move large distances, it is reasonable to expect frequent movement and possible gene flow between isolated seagrass meadows. In fact the dugong's reliance on obtaining adequate seagrass resources to sustain itself could be the incentive for some of the movements observed in satellite tracking studies (Marsh *et al.,* 2004).

Historically a major barrier to gene flow among dugong populations would have been land bridges that appeared during periods of low sea level. For example, a land bridge between Papua New Guinea and Northern Australia was thought to be present and stable until the Pleistocene, when the sea levels began to fluctuate due to the beginning of the glacial cycles (Doutch, 1972). Sea level was variable through much of the Pleistocene, with Australia and New Guinea being joined and separated repeatedly throughout this period (Galloway and Loffler, 1972; Keenan, 1994). It is thought that the land bridge between Northern Australia and Papua New Guinea most recently submerged between 7 000 and 11 000 years before the present (Jennings, 1972). These repeated changes in sea level would obviously result in repeated changes in the extent of shallow waters over the continental shelf around Northern Australia and would have a major impact upon the coastal ecological communities of the region. During the last glacial maximum, much of the North Australian shoreline would have been located on the much steeper continental rise (Galloway and Loffler, 1972) where suitable sites for seagrass meadows would not have existed - severely limiting the available dugong habitat. If dugongs inhabited Australian waters during this period, long distance movements would have been inhibited or even prevented by lack of habitat and/or land bridges.

### *4.1.4 Aim of chapter*

Tikel (1997) presented the first evidence of phylogeographic divisions in the dugong. Analysing sequence data (198 bp) from the 5' domain of the mtDNA control region from 103 individuals, she identified two distinct lineages within Australia that overlapped geographically in the Great Barrier Reef region. This phylogeographic division is surprising given that dugongs are large mammals that have been shown to

travel considerable distances (see above). Here, I present data from a longer segment of the control region with a larger, geographically more representative collection of samples including many from Northern and Western populations of Australian dugongs. These additional data allow a better understanding of the processes that have shaped the present distribution of the dugong in Australia and the testing of the hypothesis that two refugia for dugongs were present on the Australian coast during the glacial maximum. My results also offer insights into current population genetic structuring, gene flow and connectivity among populations. This additional data will aid in attempts to infer dugong behaviour responsible for the patterns observed, thus providing essential information for planning the future management of the species.

## 4.2 Methods

### *4.2.1 Sample collection, DNA extraction, PCR and sequencing*

Samples were obtained from dugongs from a variety of sources from almost the full extent of the dugong's range in Australia (Appendix 4): dead stranded animals, animals taken by Indigenous hunters, biopsies from live animals used for satellite-tagging experiments, and biopsies taken from free-ranging animals using a scraping device designed by Tikel (1997). DNA extraction followed van Oppen *et al.* (1999) (see section 3.2.2).

Primers used for the amplification of a 500 base pair segment of the mtDNA control region were those designed by Kocher *et al.* (1989) (A24 - 5' end) and Tikel (1997) (A58 - 3' end) (see Table 3.2). The 5' primer is positioned in the $tRNA^{pro}$ and the 3' in the central conserved domain of the control region (see Figure 3.1). These primers were also used as sequencing primers. On publication of the entire dugong mitochondrial genome (Murata *et al.*, 2003) it was noted that these primers matched poorly at the 5' end, hence further studies should redesign primers specific for the dugong control region. Amplification was conducted using Qiagen *Taq* PCR reagents in the following concentrations per 25 µL reaction: 1x PCR buffer, 2 mM $MgCl_2$, 0.16 mM dNTPs, 1x Q solution, and 1 unit of *Taq* DNA polymerase. The following PCR profile was used: an initial step of 5 min at 96ºC followed by 30 cycles of 30 s at 96ºC, 30 s at 50ºC, 1 min at 72ºC, followed by a final step of 10 min at 72ºC.

The PCR products were excised from an 1% agarose gel containing 40 mM Tris-acetate, 1 mM EDTA and purified using a QIAquick gel purification kit (Qiagen Catalogue Number 28704) following the manufacturer's instructions. DNA sequencing used ABI Big Dye chemistry (Applied Biosystems catalogue number 430315509197) and was run on ABI377 sequencer. Sequence traces were viewed and checked using Sequencher 3.1.1 (GeneCodes, 1991) and aligned in Se-Al v1.0a1 (Rambaut, 1996).

### *4.2.2 Groupings of samples and haplotype identification*

Sequences from a preliminary study on the phylogeography of dugongs (Tikel, 1997), employing a 198 bp fragment of the 5' end of the control region were also used in this study. Due to the difference in length of the two fragments a number of the analyses were carried out on the shorter region (198 bp – Tikel's data and my data) and the longer region (492 bp - my data) separately. Haplotypes were identified in the two data sets using Sequencher 3.1.1 (GeneCodes, 1991). Each unique haplotype was given an identifying number. For haplotypes that had been identified by Tikel (1997) the code DT was placed before the number.

Because of the difficulty of obtaining samples from some specific locations, analyses were conducted on regional groupings (Western Australia, Northern Australia, Northern Queensland, Southern Queensland, and outside Australia; Figure 4.1, Appendix 4, section 2.1.1). This grouping of populations is concordant with recent data from aerial surveys, indicating that although large scale movement occurs between and within survey areas of tens of thousands of square kilometres, there is no evidence of such movements at the scale of the Australian range as a whole (Marsh *et al.,* 1997; Marsh and Lawler, 2001, 2002; Marsh *et al.*, 2004; Gales *et al.*, 2004). My results revealed two major lineages around Australia (widespread lineage and restricted lineage) and indicated that additional lineages occur in other countries. In order to try to elucidate the processes occurring within these lineages, some analyses were conducted on each lineage separately as well as the total data set and regional groups. For definitions of populations refer to section 2.1.1.

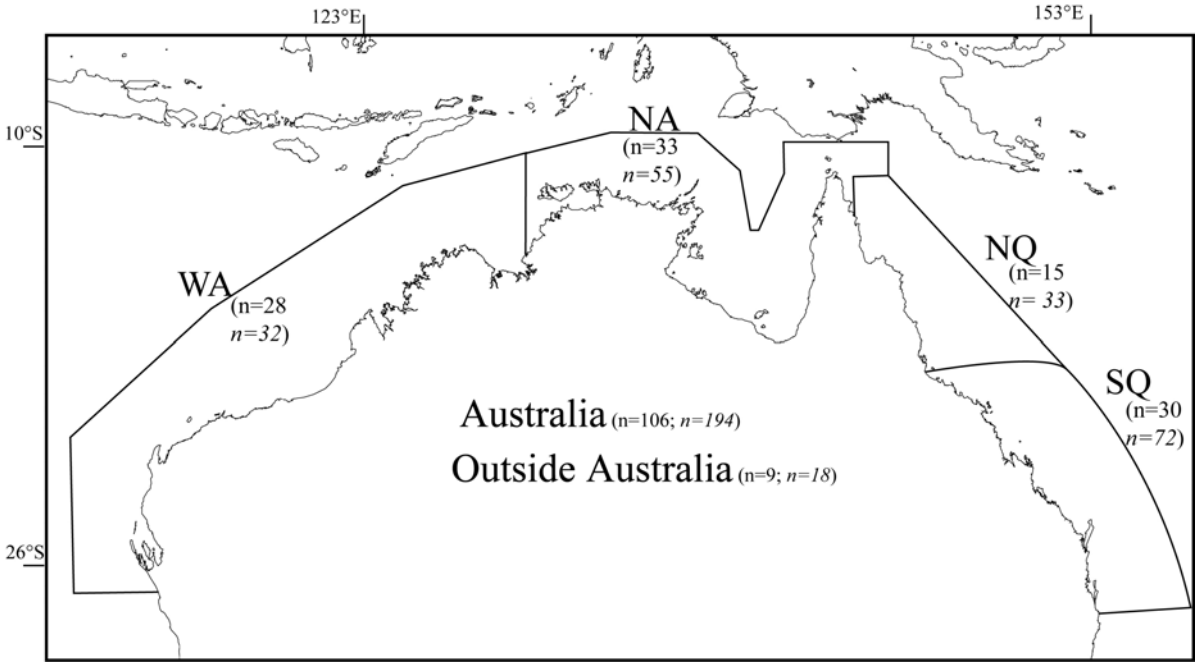



**Figure 4.1** Map indicating the regions into which samples were grouped for analyses. WA = Western Australia; NA = Northern Australia; NQ = North Queensland; SQ = South Queensland. Number of samples (n) in each region is also indicated. The number of samples (n) in the dataset including sequences by Tikel (1997) are in italics.

A number of different analyses were conducted on the data to try to elucidate the patterns and the processes that had produced them. Simple diversity indices were calculated to allow comparison with other studies (section 4.2.3). A visual way to show the relationships between the different haplotypes is by minimum spanning trees and networks (section 4.2.4). The nested clade analysis (section 4.2.5) then uses the networks to differentiate between historical and contemporary processes. Phylogenetic trees (section 4.2.6) are another convenient and commonly used way of depicting the relationships among sequences. An analysis of molecular variance (AMOVA) (section 4.2.7) identifies the amount of variation present at the different levels of hierarchical grouping, and the amount of gene flow occurring between the groups. Isolation-by-distance (section 4.2.8), identifies if the genetic diversity is correlated with geographic distance. The historical effective population size (section 4.2.9), and estimates of divergence times (section 4.2.10) were also calculated.

### *4.2.3 Diversity indexes*

The divergences within and between the lineages and regions were calculated using the distance measure in MEGA 2 (Kumar *et al.*, 2001), with the complete deletion option for missing data. The Kimura 2 parameter (K2P) algorithm was used for all divergence estimates as this allows direct comparisons between this study and other published studies. Haplotype diversity was calculated using the following equation: (equation 8.4) (Nei, 1987)

$$h = \frac{n\,(1 - \sum x_i^2)}{n - 1}$$

where, $n$ is the number of individuals in the population and $x_i$ represents the population frequency of the ith haplotype. Nucleotide diversity was calculated in ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000) using the equation of Tajima (1983) and Nei (1987).

### *4.2.4 Minimum spanning trees*

In order to identify the relationships between haplotypes, a minimum spanning analysis was conducted using ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000) on both datasets. The minimum spanning trees were then drawn (using Adobe Illustrator v10 – Adobe Systems Incorporated) based on tree distance calculated in Arlequin. As many unique haplotypes were lost when the shorter sequences were used, all further analyses were conducted on the alignment of the longer sequences only. Additional minimum spanning networks were drawn to show the relationship between haplotypes in each regional group for the longer sequences.

### *4.2.5 Nested clade analysis*

The program TCS (Clement *et al.*, 2000) was used to generate a haplotype network displaying, as ancestral or missing haplotypes, the number of base pair differences between haplotypes. This program incorporates the cladogram estimation algorithm described by Templeton *et al.* (1992), and provides all branch connections (at a 95% parsimonious level) between haplotypes. The nested clade structure of this cladogram was determined by using the procedure outlined in Templeton and Sing (1993). A nested clade analysis was then performed using the program GeoDis (Posada *et al.*,

2000) which differentiates between historical and contemporary processes by incorporating the methods outlined in Templeton *et al.* (1995). This program calculates the average distance of individuals in clade X from the geographical centre of that clade (*i.e.*, the clade distance [$D_c(X)$]) and the average distance of the clade X individuals from the geographical centre of the higher level clade in which clade X is nested (*i.e.*, the nested clade distance [$D_n(X)$]). The distances between the tip and interior clades within the nested group and the tip to interior distance for the nesting clade are also calculated. The null hypothesis of no association of clades with geographical location is tested by a permutation procedure with 1000 resamples. If the probability is less than 0.05 then a significant association between clade and geographic location has been detected. The program output and clade significance was interpreted using the key presented in Templeton *et al.* (1995).

The usefulness of the results from the above analysis is unclear as a result of the small sample size for some localities. The nested clade analysis works on the frequency of observed haplotypes — the smaller the sample size the less likely it is that uncommon haplotypes will be observed which may result in biases in the results. However, with this in mind the results detailed below are in agreement with the other analyses that were conducted.

### *4.2.6 Phylogenetic analyses*

Modeltest (Posada and Crandall, 1998) was used to estimate the most likely DNA substitution model to be used with a maximum likelihood analysis in PAUP v4.0b10 (Swofford, 2000) for the alignment of longer sequences. Maximum likelihood (using HKY85 + G + I substitution model with parameters estimated from the data by model test) and parsimony analyses were run using PAUP v4.0b10 (Swofford, 2000) for both data sets. Parsimony searches were conducted using the Goloboff correction factor (K = 2). The parsimony bootstrap analysis (500 replicates) was conducted on a conserved dataset where all missing and constant characters were excluded. The parsimony heuristic and maximum likelihood analyses were conducted on the full dataset of 492 bp.

### *4.2.7 Analysis of molecular variance*

Analyses of molecular variance were conducted, to assess the extent of gene flow between locations, and genetic variation within and among populations (Excoffier *et al.*, 1992). The genetic distance measure used was the pairwise distance function in ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000). Due to the distinct lineages that are present within the Australian dugongs as a result of a historical process, the main assumptions of the AMOVA are not met: the equilibrium assumptions of $F_{st}$-based gene flow measurements. For this reason the AMOVA was then conducted on the two lineages within Australia separately. Several different manipulations of the population groupings and hierarchical structure were conducted to verify the population subdivisions. These groupings were:

1) All populations treated as one group to test for the presence of a single panmictic population,

2) The two Australian lineages (widespread and restricted) each treated as a separate group, regardless of the population of origin of individuals within it - overseas samples were considered a further separate lineage,

3) All individuals placed in 5 regional groups (Western Australia, South Queensland, North Queensland, Northern Australia and Overseas) regardless of lineage,

4) Individuals within the restricted Australian lineage placed into the regional groups (South Queensland, North Queensland, and Northern Australia),

5) All individuals in the restricted Australian lineage considered as one group regardless of population of origin,

6) Individuals in the widespread Australian lineage placed into regional groups (South Queensland, North Queensland, Northern Australia, and Western Australia),

7) All individuals in the widespread Australian lineage considered as one group regardless of population of origin.

### *4.2.8 Isolation-by-distance*

I tested for isolation-by-distance using IBD 1.2 (Bohonak, 2002) for the Australian population as a whole and then within the identified maternal lineages of Australian

dugongs. The shoreline distance between populations in km was estimated manually with a map. These distances were the geographical distance used in this analysis. Where samples from a large geographical area were grouped as one locality, the distance measure was taken from the centre. The population pairwise $F_{st}$ calculated from ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000) using the formula from Reynolds *et al.*, (1983) and Slatkin (1995) was the genetic measure used to allow comparisons with microsatellite data also collected from these individuals (see Chapter 5). Negative $F_{st}$s were identified in a number of populations as result of using haplotypic data. For these analyses the negative genetic distances were used without transformation in the first analyses, but were set to 0.000100 when determining the log genetic distances, as negative numbers can not be log transformed.

### *4.2.9 Calculation of effective population size and population growth models*

Migrate v2.0.6 (Beerli and Felsenstein, 1999, 2001) was used to estimate theta (θ), a measure of genetic diversity, for both the widespread and restricted lineages separately. This value was then used with the d-loop substitution rate of $2x10^{-8}bp^{-1}yr^{-1}$ from Roman and Palumbi (2003), estimated in whales, to calculate the historical effective female population size using the following formula $\theta = 2N_{ef}\mu$ where $\mu$ is the substitution rate per generation. The mutation rate of $2x10^{-8}bp^{-1}yr^{-1}$ is the same as the rough estimate produced for dugongs by Tikel (1997) used in the divergence estimates below. Coalescence-based modelling on both the widespread and restricted lineage was carried out in DnaSP v4.0 (Rozas *et al.*, 2003) to try to establish whether a model of population growth/decline or of long-term population stability better fit the data. Graphs of the haplotype pairwise mismatch distribution were also produced from DnaSpV4.0 (Rozas *et al.*, 2003). Additional statistics calculated in DnaSpV4.0 (Rozas *et al.*, 2003) included Tajima's D, Fu & Li's D*, Fu & Li's F*, and Fu's Fs.

### *4.2.10 Time of divergence estimates*

The time of divergence between dugong lineages was estimated using a variety of published mutation rates for the mitochondrial control region: 1% per Myr for baleen whales (Hoelzel *et al.*, 1991; Baker *et al.*, 1993), 8% to 15% per Myr for terrestrial mammals (Vigilant *et al.*, 1991; Stewart and Baker, 1994), 2.8% per Myr for elephants

(Eggert *et al.*, 2002) and 2% per Myr for dugongs (Tikel, 1997). The divergence estimate for the dugong lineages that was used is the Kimura 2-parameter net divergence based on my dataset (492 bp; 115 individuals).

## 4.3 Results

### *4.3.1 Haplotypes and diversity indices*

From the 115 samples I sequenced in this study a total of 52 haplotypes were identified. However, fewer than half of these haplotypes (19) were found in more than one individual. From the 492 bp sequenced there was a total of 59 variable sites (Table 4.1; Appendix 5). If I include Tikel's (1997) data, then 55 haplotypes are identified with 47 polymorphic sites. However, a number of haplotypes I identified collapsed into a more common haplotype when Tikel's shorter sequences were used (Table 4.1; Appendix 6).

The dugong haplotypic diversity (*h*) calculated for my data set (115 samples – 492 bp) is high at 0.96, and slightly lower for the dataset including Tikel's (1997) samples (*h* = 0.92) (Table 4.1). Of the two lineages identified in this study, termed widespread and restricted, the widespread lineage has *h* similar to that of the dugong population as a whole (*h* = 0.95, n = 64), while the restricted lineage has an *h* of 0.87 (n = 39) (calculated from my dataset of 115 samples – 492 bp). The results of the two lineages based on the dataset containing larger sample size but shorter sequences show lower *h* and higher nucleotide diversity ($\pi$) than that based on the longer sequences (Table 4.1). The following regional diversities were calculated from the dataset generated in this study (115 individuals and 492 bp). Out of the identified dugong regional areas in Australia, Northern Australia and Western Australia have the highest *h* (0.94, and 0.93 respectively; Table 4.1), with the lowest *h* occurring in Northern Queensland (h = 0.76, Table 4.1) based on the longer sequences. The samples from outside Australia have a high *h* of 0.95. However, this is a consequence of the small sample size obtained opportunistically over a large geographic area (Table 4.1). When the dataset containing the Tikel (1997) sequences is examined, the *h* within the regions is lower compared with my dataset with the exception of North Queensland, which is higher (Table 4.1). The most significant difference in *h* between the two datasets is the South Queensland region where *h* drops from 0.86 with long sequences to 0.60 when the shorter sequences

are used (Table 4.1). Interestingly a higher number of haplotypes and higher π are identified in all regions except Western Australia with the shorter haplotypes (Table 4.1). A lower number of polymorphic sites are identified for all regions with the shorter haplotypes except for Western Australia and samples from outside Australia (Table 4.1). The genetic divergence between the widespread and restricted lineages is 1.7 – 3.5% for the long haplotypes and 0.5 – 6.8% for the short haplotypes as calculated from individual pairwise genetic distance (K2P) comparisons.

**Table 4.1** Estimates of genetic diversity based on the 5' end of the control region of the mtDNA for the regional dugong populations. Major lineages identified in this study are indicated in upper case letters. The upper row of values for each region is based on the 492 bp region sequenced in this study. Values in bold (lower row) include Tikel's (1997) data and are based on a 194 bp region. For the analysis of the short region Ashmore Reef samples were included in the Western Australia regional grouping.

| Region | N | No. Haplotypes | Haplotypic Diversity *h* | Nucleotide Diversity π (%) (±SD%) | No. Polymorphic Sites |
|---|---|---|---|---|---|
| South Queensland | 30 | 13 | 0.86 | 1.54 (±0.82) | 26 |
| | **72** | **20** | **0.60** | **2.73 (±1.48)** | **24** |
| North Queensland | 15 | 6 | 0.76 | 0.86 (±0.51) | 21 |
| | **33** | **10** | **0.77** | **2.42 (±1.35)** | **17** |
| North Australia | 33 | 18 | 0.94 | 1.96 (±1.27) | 28 |
| | **55** | **19** | **0.86** | **3.44 (±18.3)** | **21** |
| Western Australia | 25 | 15 | 0.93 | 0.48 (±0.30) | 10 |
| | **29** | **12** | **0.83** | **1.46 (±0.88)** | **17** |
| Ashmore Reef | 3 | 2 | 0.67 | 2.31(±1.81) | 17 |
| | - | - | - | - | - |
| Outside Australia | 9 | 8 | 0.97 | 1.78 (±1.03) | 20 |
| | **18** | **11** | **0.94** | **4.31 (±2.34)** | **24** |
| WIDESPREAD | 64 | 28 | 0.95 | 0.69 (±0.40) | 19 |
| | **106** | **27** | **0.88** | **1.26 (±0.76)** | **21** |
| RESTRICTED | 39 | 14 | 0.87 | 0.46 (±0.29) | 13 |
| | **86** | **15** | **0.71** | **0.66 (±0.46)** | **15** |
| OVERALL | 115 | 52 | 0.97 | 2.29 (±1.16) | 59 |
| | **212** | **54** | **0.92** | **4.01 (±2.07)** | **47** |

### *4.3.2 Minimum spanning trees and nested clade analyses*

The presence of the two lineages around Australia is clearly shown in the minimum spanning trees (MST) produced for both data sets (Figures 4.2 and 4.3). However, the distinctiveness of the lineages appears reduced in the MST produced with the shorter sequences compared with the long sequences (Figure 4.2 and 4.3). This result is due to

several haplotypes dissolving once the shorter sequences were used as clearly illustrated in Figure 4.2, which has a number of large circles, each one representing haplotypes that occur in a large number of individuals (max 39 individuals with one haplotype; Figure 4.2). The MST of the long sequences (n = 115; 492 bp) allows for a clearer interpretation of the data (Figure 4.3). For this reason all additional analyses were conducted only on the long sequence dataset. The higher resolution of the relationships between haplotypes observed when the longer sequences are used also allows the geographic overlap of the two Australian lineages to be observed with the North Australian haplotypes appearing almost evenly between the two lineages (shown in green on Figure 4.3). The positioning of Ashmore reef samples (shown in black on Figure 4.3) is also worth noting. Two samples clustered with the samples from outside Australia and one individual had the same haplotype as a Western Australian sample (Figure 4.3).

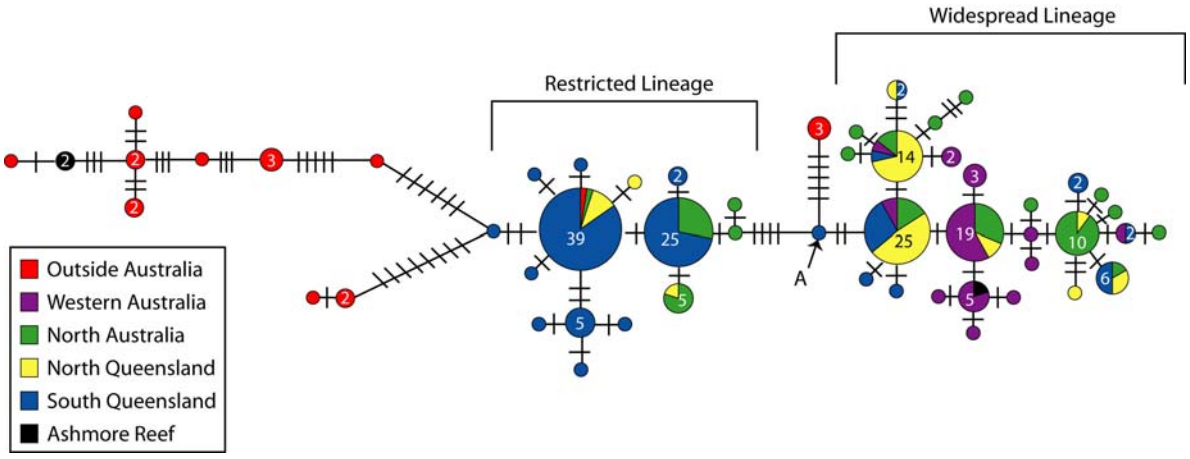



**Figure 4.2** Minimum spanning tree showing the relationships between the short haplotypes (194 bp; 212 individuals). Colours indicate the location of samples containing that haplotype. The size of each circle and the number within it indicates the number of individuals with that haplotype. Haplotypes that occur in a single individual are not numbered. Dashes indicate single base pair differences between haplotypes. 'A' indicates haplotype 34 discussed in the text. The two Australian lineages are identified.

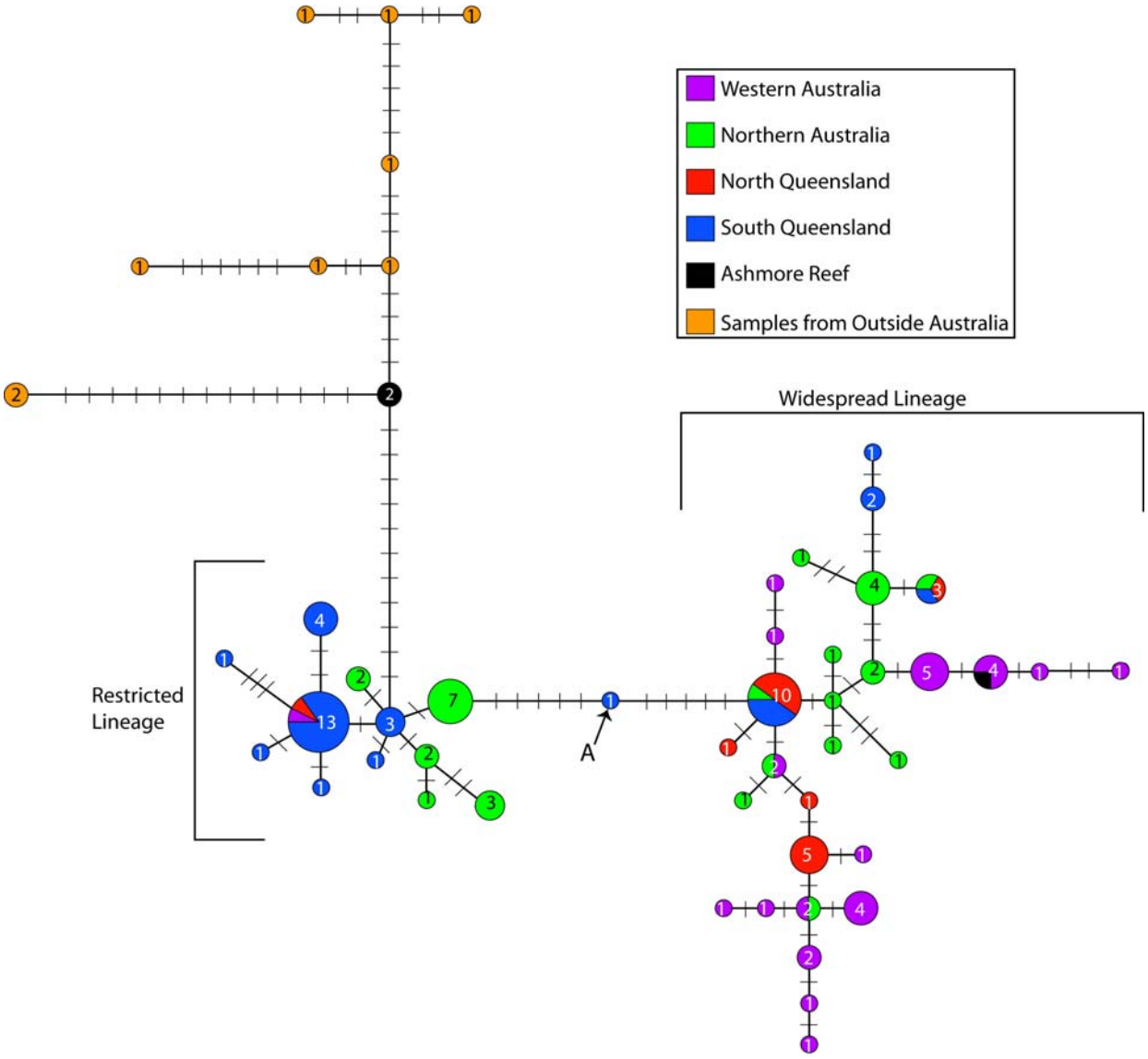



**Figure 4.3** Minimum spanning tree showing the relationships between the long haplotypes (492 bp; 115 individuals). Colours indicate the location of samples containing that haplotype. The size of each circle and the number within it indicates the number of individuals with that haplotype. Dashes indicate single base pair differences between haplotypes. 'A' indicates haplotype 34 discussed in the text. The two Australian lineages are identified.

The regional minimum spanning networks (MSN) clearly show the geographical overlap of the two lineages within Australia, with both present in the North Queensland and Northern Australian networks (Figure 4.4A and D). The MSN of the Western Australia samples clearly only contains representatives from one lineage, with a number of common closely related haplotypes surrounded by less common divergent haplotypes (Figure 4.4B). Both lineages are present in the South Queensland regional MSN with a

long divergent branch extending from a common haplotype (Figure 4.4C). The common haplotype is also surrounded by less common divergent haplotypes in a star pattern (Figure 4.4C).

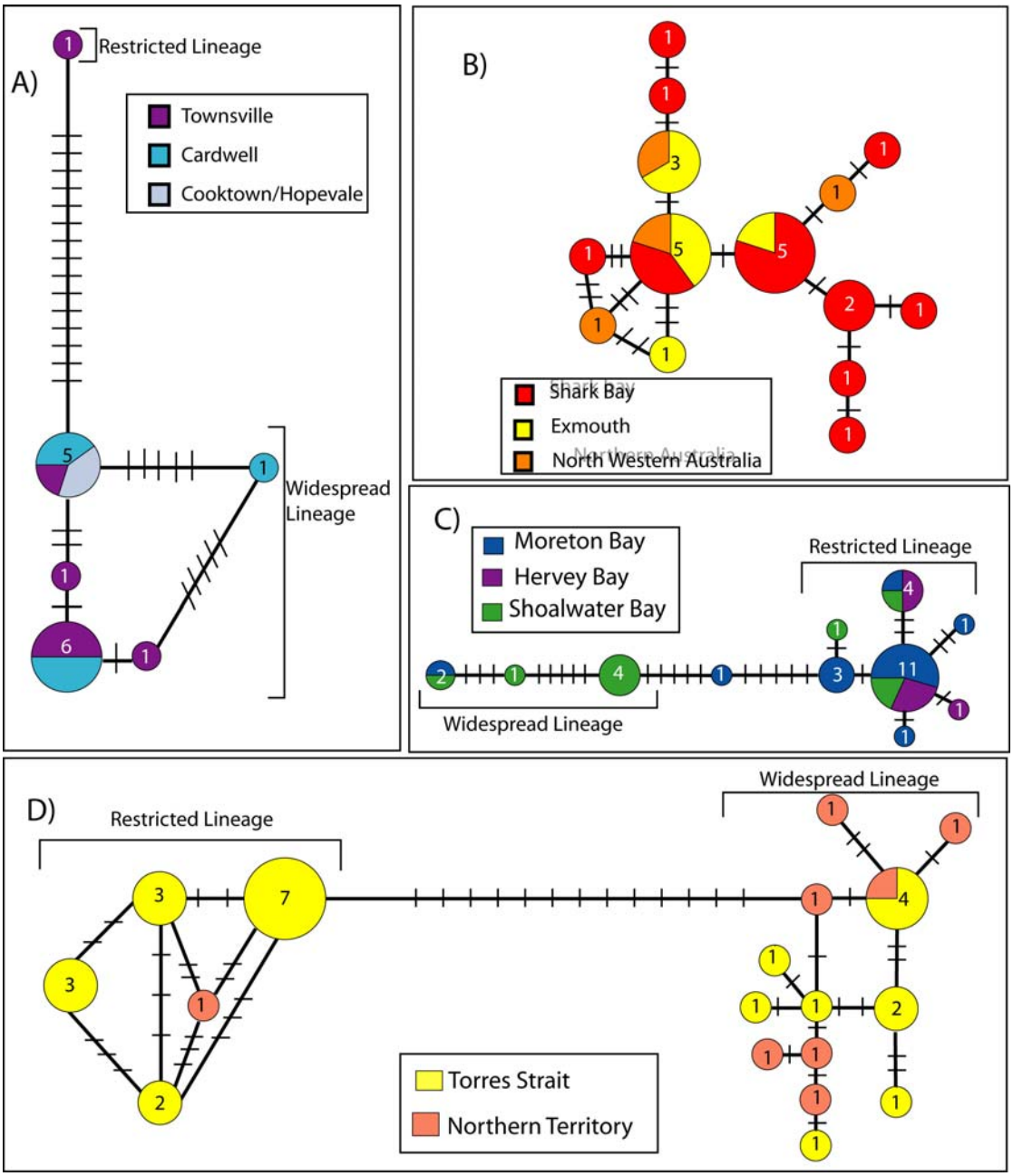



**Figure 4.4** Minimum spanning networks indicating the relationships between haplotypes within each region. The colour of the circle indicates the location of samples containing that haplotype. The size of the circle and number within it indicates the number of individuals with that haplotype. It is worth noting that all regions except for the Western Australian region (B) contain haplotypes from both Australian lineages, as indicated. A) North Queensland, B) Western Australia, C) South Queensland, and D) North Australia.

A number of significant groupings were identified in the nested clade analyses (Table 4.2; Figure 4.5), which predicts the processes occurring within the identified Australian lineages. In the restricted lineage, restricted gene flow with isolation-by-distance was the outcome of analysis at the highest grouping involving all the samples from this lineage (clade 4-1), and at a lower level between Moreton Bay, Shoalwater Bay and Torres Strait (clade 3-2; Figure 4.5). Contiguous range expansion was inferred to have been the process acting on samples from Moreton Bay, Hervey Bay, Shoalwater Bay, North Queensland and the Northern Territory (clade 3-1; Figure 4.5). The most important process in the widespread lineage appears to be range expansion (Table 4.2). This conclusion was inferred at a number of different nesting levels, from level two to the highest clustering level. Contiguous range expansion was inferred over the entire widespread lineage (clade 4-2; Figure 4.5), with a smaller clade suggesting range expansion aided by long distance colonisation between Western Australia, Northern Australia, North Queensland and some Southern Queensland samples (clade 3-4; Figure 4.5). Restricted gene flow, but with some long distance dispersal, was suggested to be responsible for the distribution of haplotypes between Western Australia, Ashmore Reef and Torres Strait (clade 2-9; Figure 4.5). Because of the position of haplotype 34 (occurring between the two lineages), the nesting tended to group with the widespread lineage a haplotype with numerous individuals from the restricted lineage. Removing it and repeating the analysis tested the importance of haplotype 34 on the interpretation. The results were similar. However, the higher-level clades were not significantly supported (data not shown), suggesting that the processes identified above and in Table 4.2 to be acting on the dugong populations in Australia may be weak. Interpretation of these findings should thus be treated with caution.

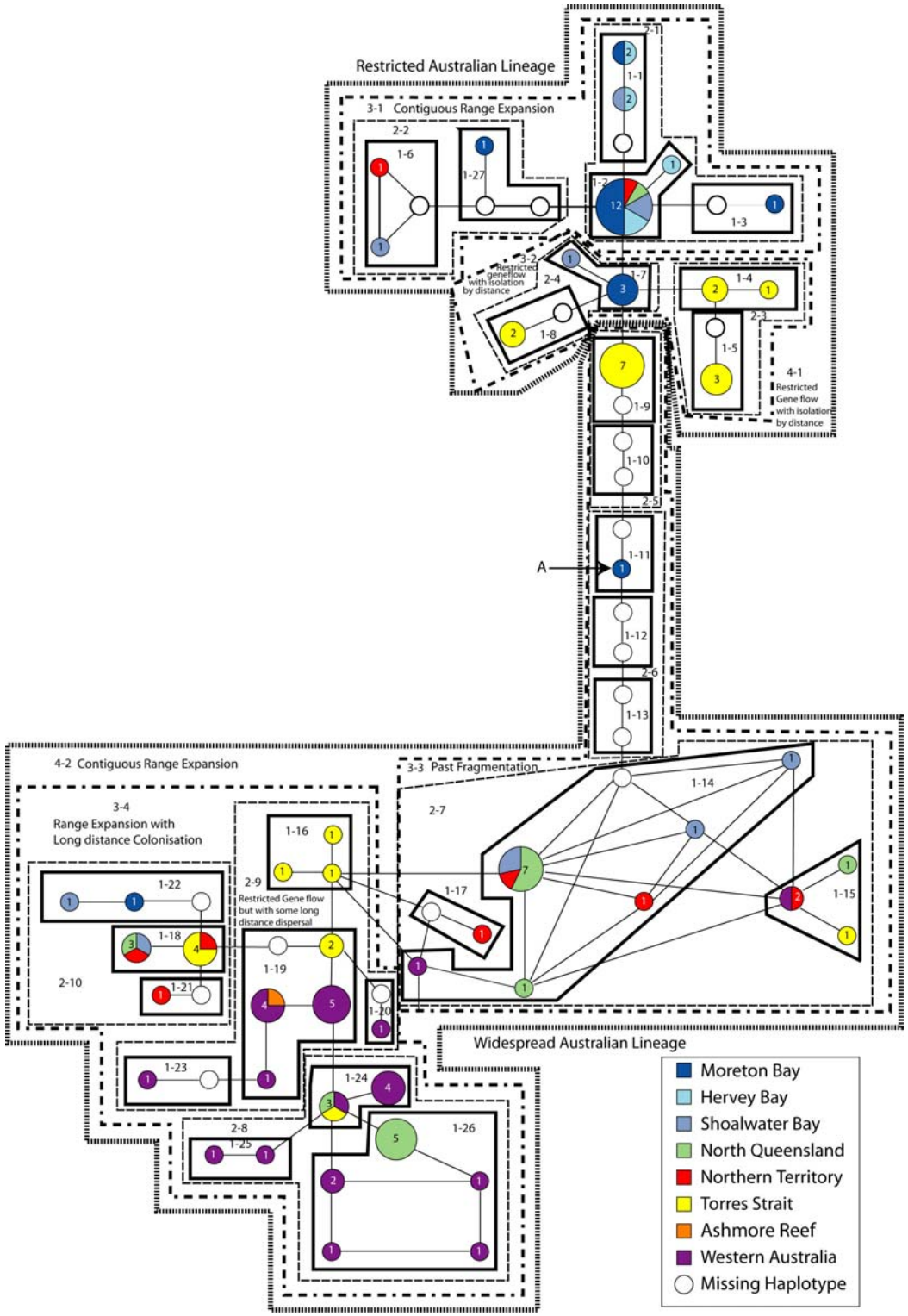



**Figure 4.5** Nested clade analysis based on 492 bp sequences of the dugong control region overlaid on the minimum spanning network from TCS. Samples from outside Australia have been removed. The first level of nesting is indicated by the bold solid line, the second by a dashed line. The third level of nesting is indicated by the dot-dash line, with the fourth and final level of nesting by the heavily dashed line. The colours indicate location of samples, with the empty circles indicating 'missing' haplotypes. The size of each circle indicates the number of individuals identified with that haplotype. Each clade is identified with a unique number preceded by a number indicating the level of nesting. Lineages are identified. 'A' indicates haplotype 34 discussed in the text.

**Table 4.2** Interpretations of the clades that have a significant association with geographic location as identified by nested clade analysis of mtDNA haplotypes of dugongs. Clades are identified in Figure 4.5. The lineage to which the clade belongs is also shown. Longitude and latitudes of sample locations are indicated in Appendix 4.

| Clade | Interpretation | Location of samples | **n** |
|---|---|---|---|
| Clade 1-19 (Widespread) | Past Fragmentation | Cape Lambert | 1 |
| | | Exmouth | 3 |
| | | Ashmore Reef | 1 |
| | | Torres Strait | 2 |
| | | Beagle Bay | 2 |
| | | Shark Bay | 3 |
| Clade 1-26 (Widespread) | Restricted dispersal/dispersal with some long distance dispersal | Cardwell | 3 |
| | | Townsville | 3 |
| | | Shark Bay | 5 |
| Clade 2-4 (Restricted) | Past Fragmentation | Torres Strait | 2 |
| | | Moreton Bay | 3 |
| | | Shoalwater Bay | 1 |
| Clade 2-9 (Widespread) | Restricted dispersal/dispersal but with some long distance dispersal | Torres Strait | 5 |
| | | Cape Lambert | 1 |
| | | Exmouth | 3 |
| | | Ashmore Reef | 1 |
| | | Beagle Bay | 2 |
| | | Shark Bay | 5 |
| Clade 3-1 (Restricted) | Range expansion: Contiguous range expansion | Moreton Bay | 9 |
| | | Hervey Bay | 6 |
| | | Shoalwater Bay | 3 |
| | | Townsville | 1 |
| | | Bluemud Bay | 1 |
| Clade 3-2 (Restricted) | Restricted gene flow with Isolation-by-distance | Torres Strait | 8 |
| | | Moreton Bay | 3 |
| | | Shoalwater Bay | 1 |
| Clade 3-3 (Widespread) | Past Fragmentation | Torres Strait | 8 |
| | | Moreton Bay | 1 |
| | | Shoalwater Bay | 4 |
| | | Cardwell | 1 |
| | | Townsville | 2 |
| | | Starcke River | 1 |
| | | Cooktown | 1 |
| | | Bluemud Bay | 2 |
| | | Roebuck Bay | 1 |
| | | Darwin | 2 |
| | | Exmouth | 1 |
| Clade 3-4 (Widespread) | Restricted gene flow/dispersal but with some long distance dispersal | Townsville | 4 |
| | | Torres Strait | 9 |
| | | Exmouth | 4 |
| | | Shark Bay | 15 |
| | | Beagle Bay | 3 |
| | | Cardwell | 4 |
| | | Baresand Island | 1 |
| | | Moreton Bay | 1 |
| | | Cape Lambert | 1 |
| | | Ashmore Reef | 1 |
| | | Darwin | 1 |
| | | Bluemud Bay | 1 |
| | | Shoalwater Bay | 2 |
| Clade 4-1 (Restricted) | Restricted gene flow with isolation-by-distance | Moreton Bay | 12 |
| | | Hervey Bay | 6 |
| | | Shoalwater Bay | 4 |
| | | Townsville | 1 |
| | | Bluemud Bay | 1 |
| | | Torres Strait | 8 |

Table 4.2 continued over page

Table 4.2 continued

| Clade | Interpretation | Location of samples | **n** |
|---|---|---|---|
| Clade 4-2 (Widespread) | Range expansion: Contiguous range expansion | Torres Strait | 17 |
| | | Moreton Bay | 2 |
| | | Shoalwater Bay | 6 |
| | | Cardwell | 5 |
| | | Townsville | 6 |
| | | Starcke River | 1 |
| | | Cooktown | 1 |
| | | Bluemud Bay | 3 |
| | | Roebuck Bay | 1 |
| | | Darwin | 3 |
| | | Exmouth | 5 |
| | | Shark Bay | 15 |
| | | Beagle Bay | 3 |
| | | Baresand island | 1 |
| | | Cape Lambert | 1 |
| | | Ashmore Reef | 1 |

### *4.3.3 Phylogenetic results*

The trees found by maximum likelihood and maximum parsimony analyses were similar with differences occurring only in the arrangement and order of samples within the major clades. Model Test indicated that a substitution model of HKY85+G+I is appropriate for this data set and estimated the parameters from the data, where HKY is based on the mutation model identified in Hasegawa *et al.* (1985), G (gamma) indicates unequal rates of substitution along the sequence (alpha = 1.094) and I is the proportion of invariable sites (0.07317). The parsimony heuristic analysis found 6744 equally most-parsimonious trees. One of these trees is shown with the bootstrap values (from 500 replicates) indicating the supported nodes (Figure 4.6A). The majority rule consensus tree of 6744 most parsimonious trees is also shown (Figure 4.6B). There is little obvious structure within each Australian lineage (Figure 4.6). One sample from Moreton Bay (Haplotype 34 in Figure 4.6) lies close to the base of the restricted lineage. The clustering of two Ashmore Reef samples with those from outside Australia was consistent in all trees constructed.

**A)**

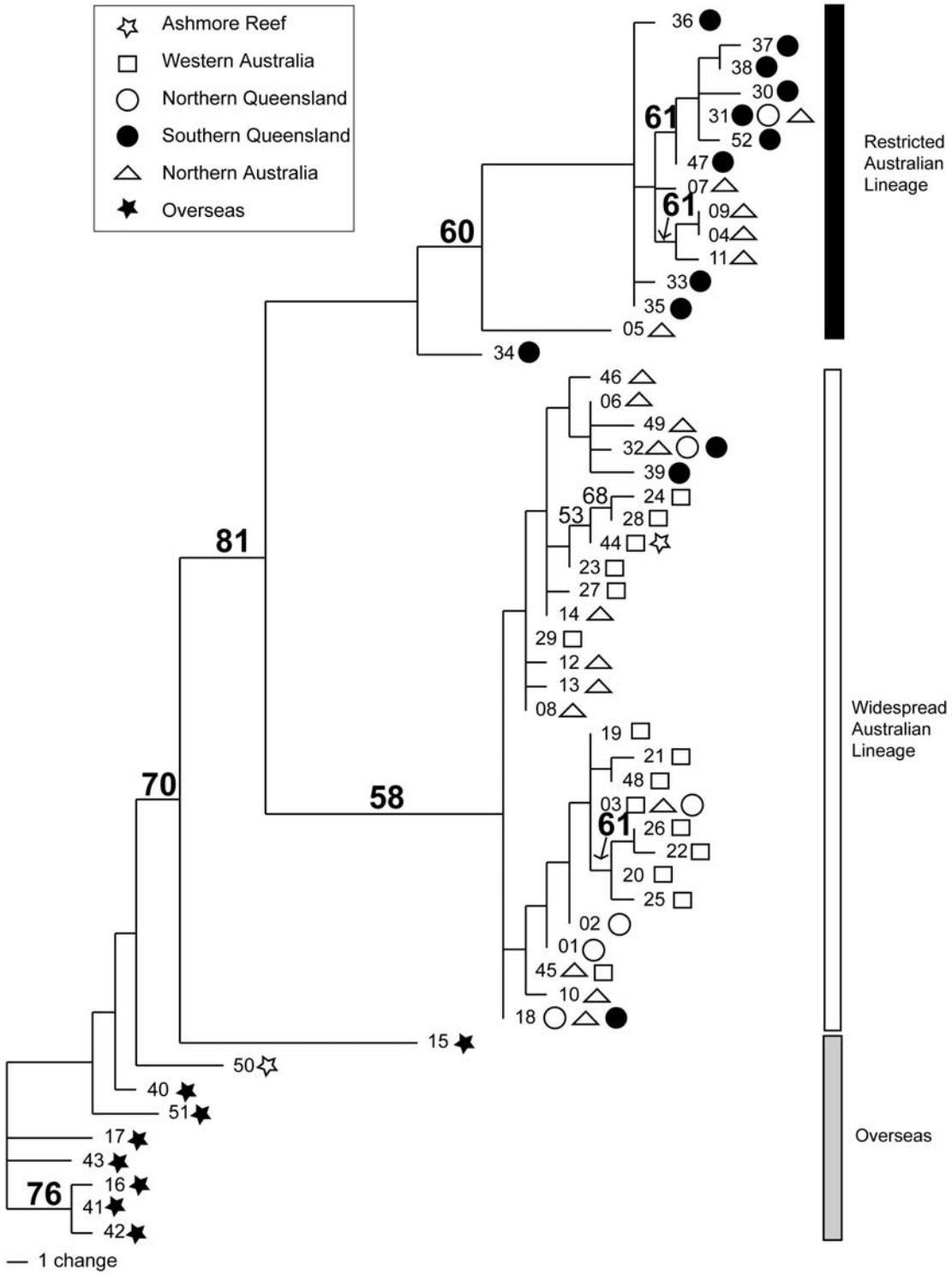



**Figure 4.6** Phylogenetic trees produced from parsimony analyses of the 492 bp sequence alignment of dugong control region. The numbers indicate the haplotype and the symbols the regions the haplotypes were found. Major clades are identified. Individuals with each haplotype are indicated in Appendix 4. Trees produced from maximum likelihood analyses have equivalent topology. A) One of 6744 equally most-parsimonious trees produced from a parsimony heuristic search. The Japan samples are the outgroup. The bootstrap values for supported nodes are indicated. B) Majority rule consensus tree of 6744 equally most parsimonious trees.

**B)**

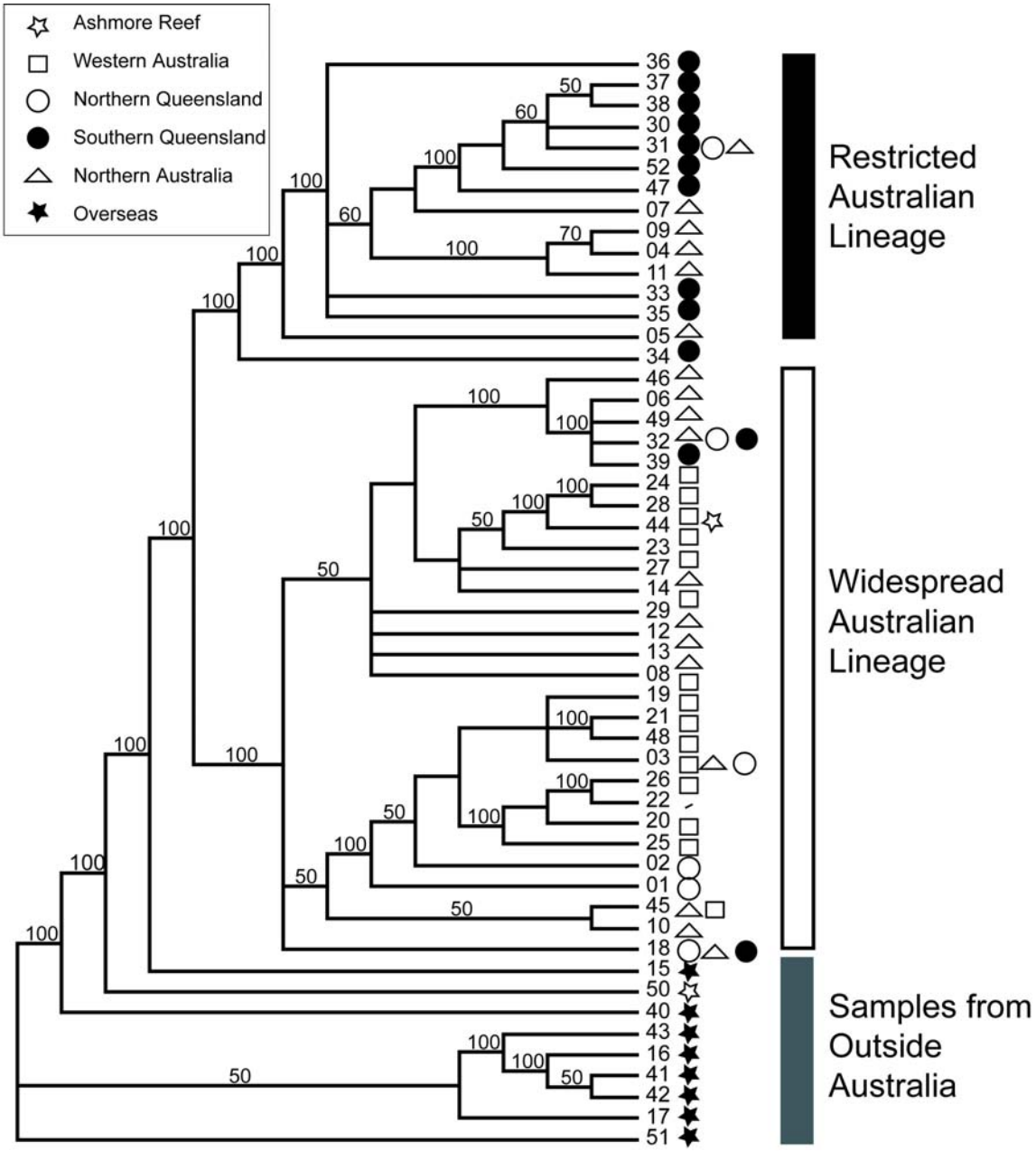



**Figure 4.6** Phylogenetic trees produced from parsimony analyses of the 492 bp sequence alignment of dugong control region. The numbers indicate the haplotype and the symbols the regions the haplotypes were found. Major clades are identified. Individuals with each haplotype are indicated in Appendix 4. Trees produced from maximum likelihood analyses have equivalent topology. A) One of 6744 equally most-parsimonious trees produced from a parsimony heuristic search. The Japan samples are the outgroup. The bootstrap values for supported nodes are indicated. B) Majority rule consensus tree of 6744 equally most parsimonious trees.

### *4.3.4 Isolation-by-distance*

A Mantel test (one sided test with 1000 randomisations) conducted using the program IBD for correlation between genetic distance (population pair-wise $F_{st}$) and geographical distance (shoreline distance in km), gave significant results for Australian dugongs, both lineages combined, (r = 0.4514, p = 0.017). There is a no significant relationship within the restricted Australian lineage (Figure 4.7A). In contrast, there is significant positive relationship (r = 0.5370, p = 0.007, Figure 4.7B) indicating some isolation-by-distance in the widespread Australian lineage.

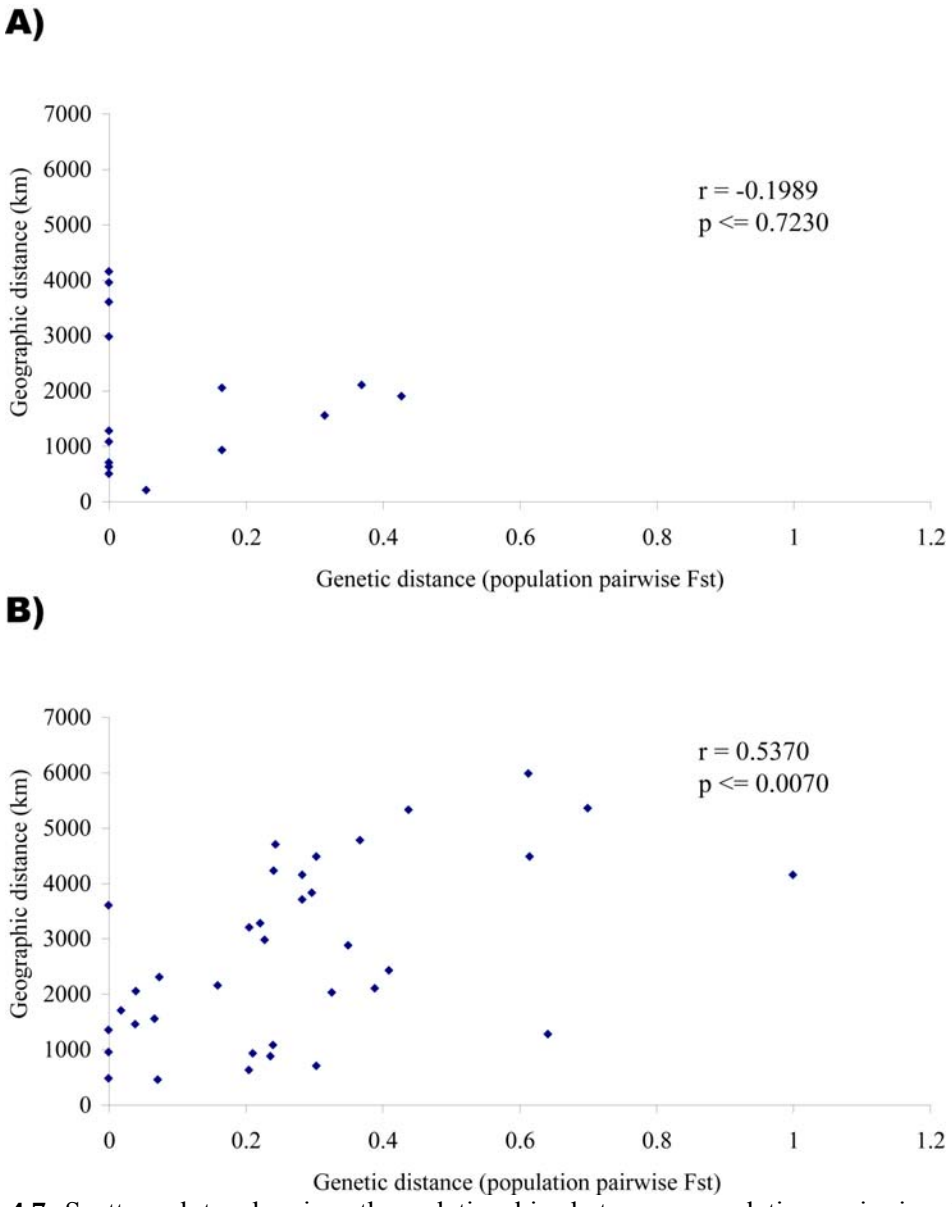



**Figure 4.7** Scatter plots showing the relationship between population pairwise $F_{st}$ and geographic distance for the two lineages within Australia. A) Restricted lineage, B) Widespread lineage.

### *4.3.5 AMOVA analyses*

As shown in Table 4.3, the hypothesis that the dugong population is panmictic is rejected at all scales examined with P values always being highly significant (p = 0.000). An AMOVA, both at the global scale ($\phi_{st}$ = 0.48) and at the Australian scale ($\phi_{st}$ = 0.83), reject panmixia (Table 4.3). In AMOVA 3, (Table 4.3) the $\phi_{sr}$ and $\phi_{st}$ values are high (0.37 and 0.50 respectively) indicating that there are limited gene flow between the suggested geographical populations and regional groupings. This result is not unexpected due to the structure already identified between the two lineages (Figure 4.3). There appears to be more gene flow within the widespread lineage than the restricted lineage with $\phi_{st}$s of 0.27 and 0.36 respectively (Table 4.3; AMOVA 4 and 6). However, this may be a product of the different sample sizes within each lineage. Most of the variation occurs among individuals within a population for both lineages (Table 4.3; AMOVA 5 and 7).

### *4.3.6 Effective population sizes and population growth/decline models*

The historical effective female population size calculated for the restricted lineage is about 17 000 (range ≈ 11 000 – 27 000), and for the widespread lineage it is about 27 000 (range ≈ 20 000 – 37 000). The actual population size is estimated to be at least six times the female effective population size (Roman and Palumbi, 2003) which would make the historical population size of the restricted lineage is about 104 000 individuals. Similarly the historical population size of the widespread lineage is estimated at 164 000 dugongs. Both the widespread and restricted lineages conform to the expectation of population growth and decline (Figure 4.8). The shape of the pairwise mismatch distribution graphs implies population growth and decline is the appropriate model for both lineages (Figure 4.8). Population growth, rather than decline, in both lineages is indicated by negative values for Tajima's D (Table 4.4). A positive value for Tajima's D indicates contraction of the population which is not indicated in either lineage here (Table 4.4). Similarly, if there is a significant value for Fu's Fs and non-significant values for F* and D* the population growth is also implied. This is the case for the restricted lineage (Table 4.4). However, the significant values for F*, D* and for Fs are contradictory in the widespread lineage, suggesting that selective sweeps or background selection might be acting on this lineage.

**Table 4.3** Analysis of Molecular Variance Analyses (AMOVA) indicating the amount of variation attributable to each hierarchal grouping, the significance and interpretation of data. Geographic localities of samples are indicated in Appendix 4. Significance tested by 1000 permutations.

| Test | Structure | % Variation | $\phi$-statistics | Significant at $p < 0.05$ level |
|---|---|---|---|---|
| 1 | All geographic locations as one group | 48%Among locations<br>52% Within locations | $\phi_{st}$ = 0.48 | Yes. Reject panmixia globally |
| 2 | Two Australian lineages as one group | 83% Between lineages<br>17% Within lineages | $\phi_{st}$ = 0.83 | Yes. Reject panmixia in Australia. |
| 3 | All geographic locations placed into 4 groups regardless of mitochondrial lineage: Western Australia, East Australia, Northern Australia and Overseas | 20.5% Among groups<br>29.8% Among locations within groups<br>49.6% Within geographic locations | $\phi_{sr}$ = 0.37<br>$\phi_{st}$ = 0.50<br>$\phi_{rt}$ = 0.20 | Yes. Reject panmixia. Most variation is at the individual level. Some structure among the groups and within the groups. |
| 4 | Restricted lineage - geographic locations Placed into 2 groups: East Australia and North Australia | 39% Between North and East Australia<br>-2.73% Among the populations within groups<br>63.43% Within geographic locations | $\phi_{sr}$ = -0.04<br>$\phi_{st}$ = 0.36<br>$\phi_{rt}$ = 0.39 | Yes. Most variation is among individuals within each population. Most structure is between the north and east coast groups |
| 5 | All geographic locations in the restricted lineage treated as one group | 27% Among geographic locations<br>73% Within geographic locations | $\phi_{st}$ = 0.27 | Yes. Most variation at individual level |
| 6 | Widespread lineage - geographic locations placed in 3 groups: Western Australia, east coast and North Australia | 17% Among groups<br>11% Among geographic locations within groups<br>72% Within geographic locations | $\phi_{sr}$ = 0.13<br>$\phi_{st}$ = 0.28<br>$\phi_{rt}$ = 0.17 | Yes. Most variation is among individuals at the population level. Less structure among groups than test 4, however more variation among the populations |
| 7 | All geographic locations in the widespread lineage as one group | 25% Among geographic locations<br>75% Within geographic locations | $\phi_{st}$ = 0.25 | Yes. Most variation at individual level |

**Table 4.4** Summary statistics for the coalescent population growth decline models in each lineage. Significant values are indicated with a '#'. Negative values for Tajima's D indicate population growth, If Fu & Li's F* and D* are not significant and Fu's Fs is then population expansion is indicated.

| Lineage | Fu & Li's D* | Fu & Li's F* | Fu's Fs | Tajima's D |
|---|---|---|---|---|
| Restricted | -2.32240 | -2.43266 | -3.811# | -1.53159 |
| Widespread | -3.26424# | -3.13616# | -10.014# | -1.54206 |

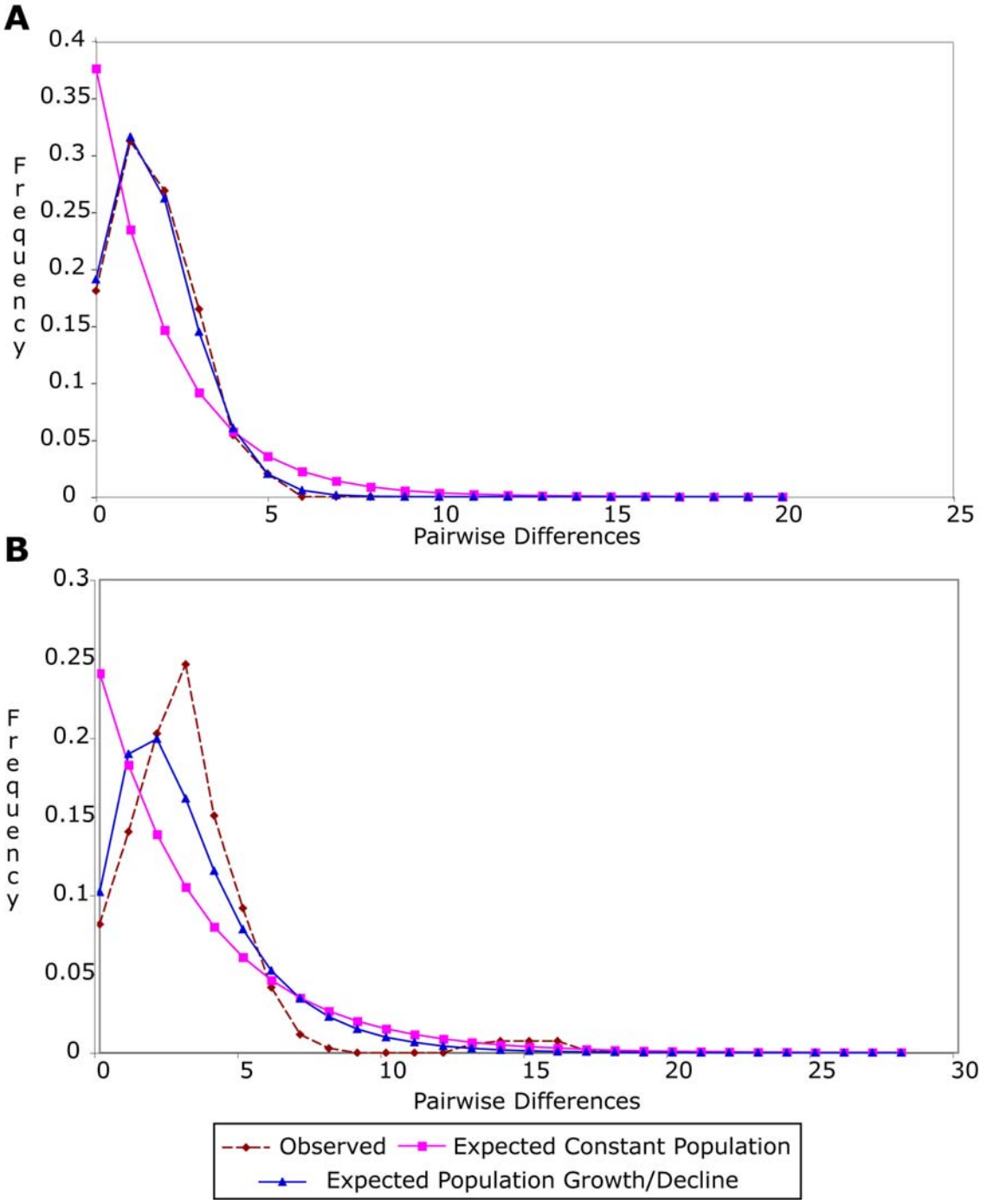



**Figure 4.8** Pairwise mismatch distribution graphs of the two Australian lineages. The distribution expected under the constant population size model and population growth/decline model are indicated. A) Restricted Australian lineage. B) Widespread Australian lineage.

### *4.3.7 Divergences times*

The estimated divergence times for the dugong lineages range from 237 500 years to 1.9 Myrs depending on the mutation rate used (Table 4.5). The estimate is of limited value due to the large range in the estimated mutation rates. The mutation rate taken from Tikel (1997) is a rough estimate due to the limited fossil record available (see section 4.1.1). However, use of this estimate does produce divergence times roughly similar to those obtained when mutation rates estimated for the Proboscidea are used. Therefore the most probable divergence time of the two Australian lineages is somewhere between 678 000 and 950 000 years before present.

**Table 4.5** Divergence estimates between the identified Australian dugong lineages based on published control region mutation rates and the mean net divergence estimated by Kimura 2-parameter method (1.9%) on the long haplotypes (492 bp; 115 samples).

| **Mutation rate (%/Myr)** | **Based on** | **Reference** | **Divergence time (years before present) (± SE)** |
|---|---|---|---|
| 8% to 15% | Terrestrial Mammals molecular and fossil record | Vigilant *et al.*, 1991; Stewart and Baker, 1994 | 8% - 237 500 (± 75 000)<br>15% - 126 666 (± 40 000) |
| 1% | Baleen whales molecular and fossil record | Hoelzel *et al.*, 1991; Baker *et al.*, 1993 | 1 900 000 (± 600 000) |
| 2.8% | African and Asian Elephant | Eggert *et al.*, 2002 | 678 571 (± 214 285) |
| 2% | Dugong fossil record | Tikel 1997 | 950 000 (± 300 000) |

## 4.4 Discussion

### *4.4.1 Phylogeography of dugongs indicate secondary contact between differentiated lineages*

The presence of two distinct mtDNA lineages (one geographically widespread, one more restricted) of dugongs within Australia has been convincingly confirmed (Figures 4.3 and 4.6). Both lineages are distinct from lineages from Asia. This phylogeographic pattern indicates the long-term isolation of the two identified Australian lineages from each other and from populations in Asia. High nucleotide diversity and haplotypic diversity, as noted for the dugong (Table 4.1), are suggested by Grant and Bowen (1998), to be indicative of a "*large stable population with long evolutionary history or*

*secondary contact between differentiated lineages*", here the second is most likely to be the more important process.

I regard the separation of Australian dugong mitochondrial lineages to be a consequence of the Torres Strait land bridge that existed periodically during periods of glacial maxima, the most recent between approximately 7 000 to 115 000 years ago (Keenan, 1994). Based on sea level reconstructions and ecological information about dugong foraging behaviour, the only suitable habitat to support the growth of seagrass, and hence of dugongs, along the east coast for most of that time would have been around the Capricorn-Bunker group in the Southern Great Barrier Reef (D. Hopley pers. com. 2002; Figure 4.9A). Water temperatures to the south of this would have been too low for dugongs at that time. It is assumed similar refugia, although larger in area and widely spaced geographically, were also present west of the land bridge (Figure 4.9A). Rising sea levels would have first produced abundant and connected shallow water habitat in the region west of the land bridge, allowing the expansion of dugong populations up to the western edge of the Torres Strait land bridge (Figures 4.9B and 4.9C). Data from Western Australian samples support this hypothesis with the high haplotypic diversity and low nucleotide diversity (Table 4.1), indicating a "*population bottleneck followed by rapid population growth and accumulation of mutations*" (Grant and Bowen 1998). The MSN of Western Australian samples also supports this hypothesis, with a number of common haplotypes surrounded by less common, more divergent haplotypes, which is indicative of a bottleneck or founder effect (Figure 4.4B). The presence of isolation-by-distance in the widespread lineage (Figure 4.7) also supports this hypothesis, as does the higher number of haplotypes and higher haplotypic diversity in the widespread lineage compared with the restricted lineage (Table 4.1). The idea of rapid divergence and increase in habitat availability on the west coast of Australia is also indicated in the contiguous range expansion suggested for the widespread lineage (Clade 4-2, Figure 4.5, Table 4.2) by the nested clade analysis.

In contrast to the situation west of the Torres Strait, dugongs in the Capricorn-Bunker refugium would have been restricted to that area until just before the final inundation of the Torres Strait land bridge approximately 7 000 years ago (Keenan 1994; Figure 4.9A - F). The mtDNA haplotypes reflect this process with lower haplotypic and

nucleotide diversity found in the restricted lineage as compared with the widespread lineage (Table 4.1). This conclusion is also supported by the MSN of Southern Queensland haplotypes belonging to the restricted lineage that shows a star pattern produced from a founder effect (Figure 4.4C).

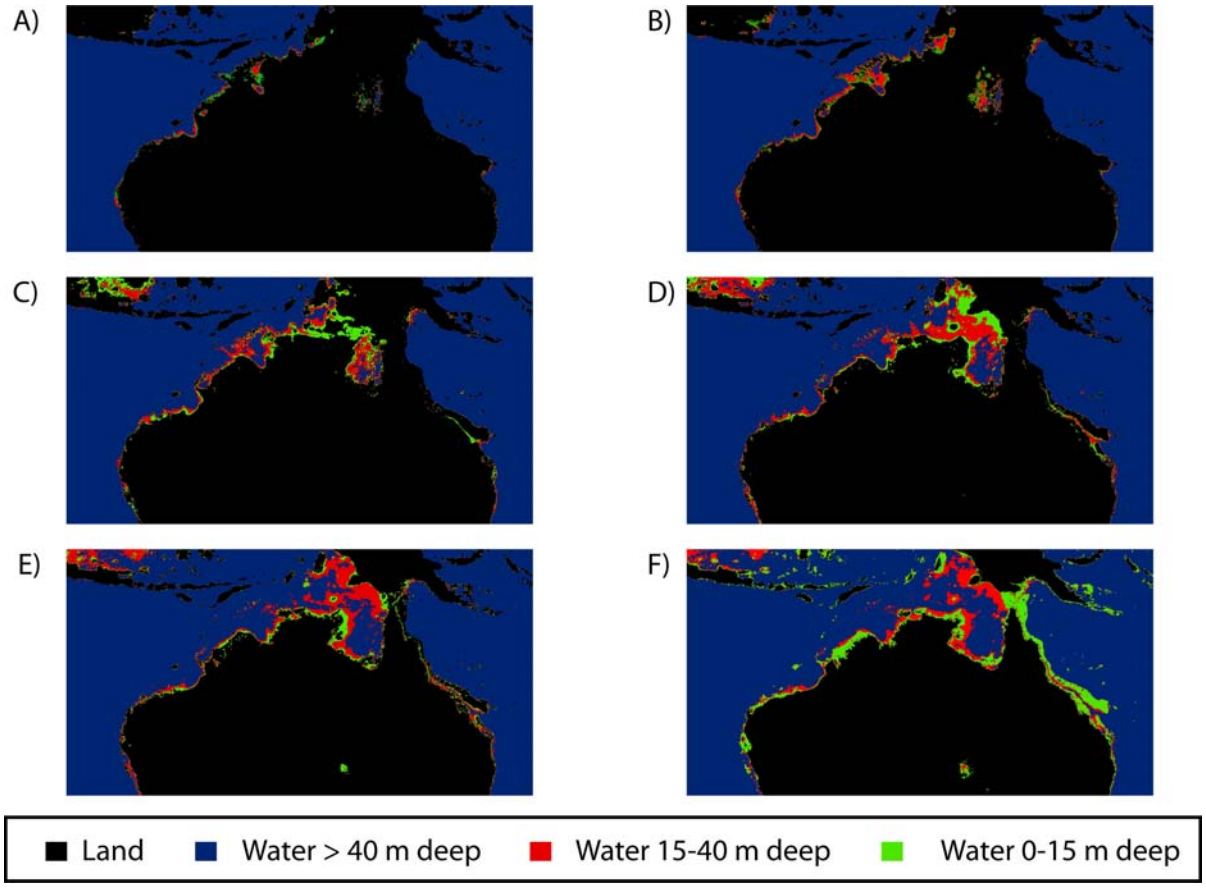



**Figure 4.9** Sea-level reconstructions produced by J. Guinotte showing the available area for dugong habitat from last glacial maximum to present day. Water depths < 15 m is suggested to be favoured dugong habitat, while water depths of 15-40 m is possible dugong habitat and waters deeper than 40 m is unlikely habitat for dugongs (Chilvers *et al.*, 2004). A) Land mass at last glacial maximum when sea level was 100 m below current sea level. B) Land mass when sea level was 75 m below current sea level. C) Land mass when sea level was 50 m below current. D) Land mass when sea level was 25 m below current. E) Land mass when sea level was 10 m below present. F) Present day land area.

The distribution mismatch and the neutrality tests for both Australian lineages (Table 4.4 and Figure 4.8) suggest that each lineage has undergone population growth at some

time in the past. The low number of pairwise differences in both lineages suggests that this growth is a recent occurrence (Figure 4.8). The calculated effective population sizes for both lineages suggest that the widespread lineage has historically had a larger population size than the restricted lineage. This result is not surprising if the widespread lineage had a large refugium (or several refugia) and more time than the restricted lineage, to expand in size and distribution as suggested above.

The most probable mutation rate is that derived from studies on elephants (2.8% per Myr) or from the fossil record of dugongs at 2% per Myr. These rates are one of the slower rates estimated for mammals and gives a divergence time between the two Australian lineages of approximately 700 000 to 1 million years (Table 4.5). This divergence time clearly far predates the most recent submergence of the Torres Strait land bridge (Table 4.5). However, the usefulness of this estimate is questionable due to the large error associated with estimates of mutation rates. In order to reduce the error rate, a mutation rate based on the mtDNA control region in Sirenia needs to be developed. This rate used in conjunction with information on the fossil record of sirenians will allow a valuable insight into when these distinct lineages diverged. Without a more significant fossil record and knowledge of mutation rate, the divergence of the Australian dugong lineages will remain difficult to estimate.

The phylogeographic partitions that are found in this study are consistent with other recorded phylogeographic breaks in Australian marine fauna including invertebrates (Johnson and Joll, 1993; Brooker *et al.*, 2000; Gopurenko and Hughes, 2002) and large pelagic fish (Wilson and Allen, 1987; Shaklee *et al.*, 1990; Keenan, 1994; Elliot, 1996; Begg *et al.*, 1998; Chenoweth *et al.*, 1998). This observed structuring of marine fauna is concordant with relevant faunal provinces around Australia. These are the Dampierian biogeographical region (Shark Bay to Cape York) and the Solanderian province (east coast of Queensland) (Wilson and Allen, 1987).

The Florida manatee also exhibits phylogeographic breaks between the Caribbean-Continental lineage and South American lineage consistent with the boundaries of marine biogeographical provinces (Garcia-Rodriguez *et al.*, 1998). That study analysed

410 bp of the mtDNA control region and identified three maternal lineages that overlapped geographically (Garcia-Rodriguez *et al.*, 1998). The distinctiveness of these lineages was attributed to phylogeographic partitions that have been bridged recently by the availability of habitat and by rare colonisation events (Garcia-Rodriguez *et al.*, 1998). However, the diversity of haplotypes (24 in 86 individuals, Garcia-Rodriguez *et al.,* 1998) is low in comparison to the dugong (52 in 115 individuals, this study). The main lineages in the Florida manatee differed by 4 to 7% (Garcia-Rodriguez *et al.*, 1998), a greater difference than that between the dugong lineages in Australia (1.7 to 3.5% - this study). Contrasting this, a recent study on the phylogeography of the Amazonian manatee (*Trichechus inunguis*) only identified one clade with a within-group sequence divergence of 1% (Cantanhede *et al.*, 2005). The Amazonian manatee also has a higher diversity of haplotypes (31 haplotypes in 68 individuals) than the Florida manatee (Cantanhede *et al.*, 2005).

### *4.4.2 Incomplete overlap of lineages suggests female philopatry in dugongs*

The lack of complete geographical mixing of these two lineages is surprising, particularly that the restricted lineage has not dispersed further afield. As outlined in section 4.1.2, dugongs have the ability to travel large distances with few barriers except open ocean. However, rare events, in which dugongs transit deep water, must occur occasionally, as demonstrated by the presence of dugongs with typically Asian haplotypes at Ashmore Reef (Figures 4.2, 4.3 and 4.6). The recent sighting of dugongs at Aldabra Atoll in the Indian Ocean for the first time in 25 years also proves that these deepwater crossings occur (Marsh *et al.*, 2002). Similarly, a recent sighting of a dugong at Cocos (Keeling) Islands (J. Hobbs pers. com. 2005), where the closest landmass is over 1 000 km away, also proves that dugongs can and do cross deepwater occasionally.

One explanation for the apparent lack of complete geographical mixing of maternal mtDNA lineages may be the occurrence of sex-biased dispersal, as has been noted for another sirenian, the Florida manatee (Garcia-Rodriguez *et al.*, 1998). However, both male and female dugongs have been recorded as travelling long distances (Marsh *et al.*, 1999; Sheppard *et al.*, in press). For the observed phylogeographic pattern to persist, female dugongs undertaking large-scale movements can not in general be giving birth

while they are travelling, although they may mate. I hypothesise that dugongs move for two reasons. The first is as a response to environmentally induced stress, such as destruction of seagrass meadows by climatic events (Preen *et al.*, 1995; Preen and Marsh, 1995; Gales *et al.*, 2004; Marsh *et al.*, 2004). Stressed and starved animals are unlikely to engage in courtship and mating and we must assume that they eventually return to their "home" area. The second is to provide their offspring with a spatial map of suitable seagrass areas. Deutsch *et al.* (2003) suggest that young Florida manatees learn their migratory patterns and seasonal ranges from their mothers.

### *4.4.3 Number of samples versus resolution of data*

This study clearly demonstrates the importance of ensuring sequence data contain a sufficient number of polymorphic sites to allow adequate resolution of patterns present in the data. Increasing the number of samples and losing a number of polymorphic sites due to shorter sequences blurred the patterns in comparison to data containing more polymorphic sites (*i.e.* longer sequences) with fewer individuals (Figures 4.2 and 4.3). Haplotypic diversity was also lost with the shorter sequences but nucleotide diversity was increased (Table 4.1). This increase in nucleotide diversity is due to the shorter sequences containing a relatively higher proportion of variable sites than the longer sequences (Table 4.1). Future studies using sequence data should carefully consider the advantages of sequence length versus number of samples sequenced as the resulting data can have different interpretations.

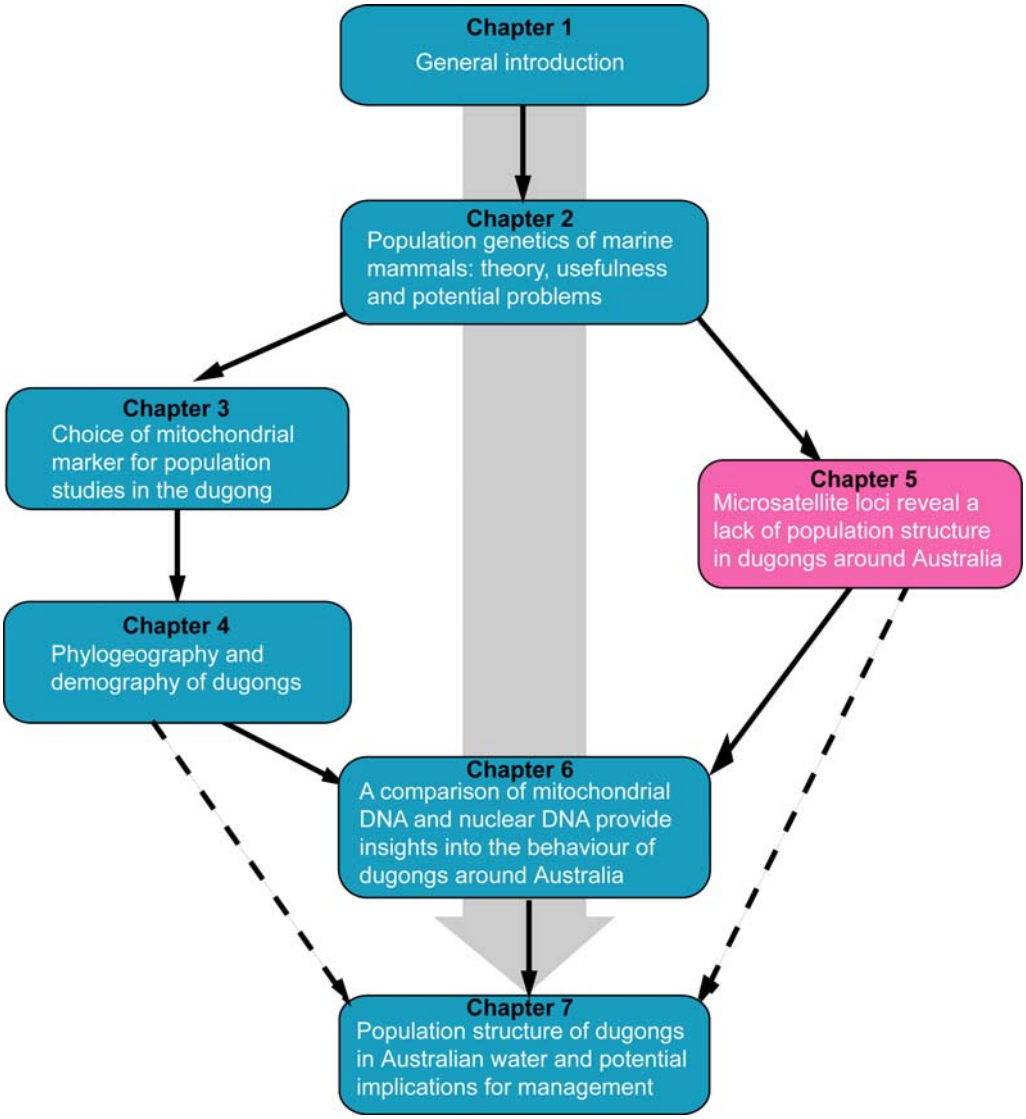
### *4.4.4 Summary of results and management implications*

The matrilines observed among Australian dugongs represent the signature of a series of vicariance events: the changes in sea level and particularly the presence of the Torres Strait land bridge for periods of much of the Pleistocene, and its recent inundation. The lack of subsequent complete geographic mixing of these lineages implies female philopatry. Regardless of why the lineages exist, these findings indicate regional-scale differences in haplotype frequency, and demonstrate that historical patterns of habitat connectivity have had a major impact upon the dugong. The distribution of seagrass beds in the coastal waters of Northern Australia is currently naturally fragmented. It is therefore likely that further fragmentation of seagrass beds around coastal Northern

Australia as a result of anthropogenic impacts will also have a significant impact on the genetic structuring of the dugong population in the longer term.

# Chapter 5: Microsatellite loci reveal a lack of population structure in dugongs around Australia

*In this chapter I evaluate the population structure of dugongs around Australia using six microsatellite loci developed for the Florida manatee. These loci displayed considerable levels of allelic diversity in the dugong and are much more variable than the Florida manatee for which the loci were developed. Analyses reveal a high level of gene flow among dugongs in Australia, combined with a significant level of isolation-by-distance. Comparisons between the mitochondrial and microsatellite data are not presented here: they are in chapter 6.*



## 5.1 Introduction

### *5.1.1 Why microsatellites are useful*

Microsatellite markers are used across a wide taxonomic range of organisms for two reasons: 1) microsatellite repeats have been found in all prokaryote and eukaryote genomes surveyed to date (Zane *et al.*, 2002) and 2) loci are easily characterised in most organisms either by developing species-specific markers or using markers developed for closely related species (Beaumont and Bruford, 1999). The usefulness of microsatellite markers is outlined in chapter 2 (section 2.5.2.6). Although microsatellites have advantages over other markers, there are still many issues that should not be overlooked. There are a number of problems associated with PCR where amplification of microsatellites are concerned. The presence of null alleles can make the scoring of microsatellites difficult. Null alleles fail to amplify because of base substitutions, insertions or deletions within the priming site (Beaumont and Bruford, 1999). The frequency of occurrence of null alleles is unknown at present and apparently varies according to the taxon studied. Stutter bands produced by slippage during replication by *Taq* DNA polymerase PCR slippage are regularly seen, especially in di-nucleotide repeats (Beaumont and Bruford, 1999). *Taq* DNA polymerase can also add an additional adenosine moiety to the 3' end of a PCR product, which can cause a single base shift in the length of microsatellite amplicon. Researchers using microsatellites need to be aware of these difficulties.

### *5.1.2 Microsatellites identified in Sirenia*

To date, only one study has identified microsatellite loci in sirenians. Garcia-Rodriguez *et al.* (2000) identified fourteen loci from a genomic library of the Florida manatee. Of the fourteen, only eight were polymorphic for the manatee. Initial screening conducted by Garcia-Rodriguez *et al.* (2000) identified nine of these markers that appeared to be polymorphic in the three dugong individuals screened.

### *5.1.3 Difficulties with using inter-specific microsatellites*

As noted previously (section 5.1.1), useful data from microsatellite loci for a species of interest may be generated through the use of loci identified in closely related species.

Homologous loci are likely to be amplified in related species using the same primers, but the percentage of amplified loci may decrease when the evolutionary divergence among species increases (Jarne and Lagoda, 1996). In addition, lower levels of allelic variation are usually detected with cross-species amplification (Frankham *et al.*, 2002). However, it is more likely that greater variation with species-specific loci will be obtained (e.g. FitzSimmons *et al.*, 1995) although not consistently (e.g. Garcia-Rodriguez *et al.*, 2000). A number of studies have identified loci that can be amplified in a range of taxa using universal primers. These include cetaceans (Schlötterer *et al.*, 1991), turtles (FitzSimmons *et al.*, 1995), and fish (Rico *et al.*, 1996). A number of loci have also been identified to be conserved across a broad range of mammals (Engel *et al.*, 1996; Moore *et al.*, 1998).

The most significant disadvantages of the *inter-specific* application of microsatellite primers are a lower level of polymorphism and poorer PCR quality, such as a higher levels of non-specific PCR products and more intense stutter bands (Estoup and Angers, 1998). A higher frequency of null alleles may also occur in loci that are isolated from other species (Estoup and Angers, 1998). In order to undertake parentage analyses and individual identification, the microsatellite loci should be highly polymorphic. Therefore species-specific loci are more likely to provide the levels of polymorphism required for such studies. However, cross-species amplification of loci may provide enough variability in certain cases to identify population structure.

### *5.1.4 Aim of chapter*

The aim of this chapter is to describe the genetic structure and generate estimates of gene flow across the dugong's range in Australia, by utilising co-dominant genetic markers. In this chapter I first describe the variability and usefulness of the manatee microsatellite loci and then the genetic structure of dugong populations in Australia. The insights generated will help us to understand the population structure, behaviour patterns and biology of the dugong.

## 5.2 Methods

### *5.2.1 Samples and DNA extraction*

In total, 452 dugong samples were screened with seven microsatellite loci originally developed for the Florida manatee (Garcia-Rodriguez *et al.*, 2000). These samples include 417 from Australian waters (Figure 5.1), 31 from Asia (Thailand, Indonesia, Philippines, Sabah, and Japan), and four from the Pacific (New Caledonia and Palau). Samples that did not amplify for more than 50% of the loci were removed from the data set, leaving a total of 372 samples that were analysed further. The non-amplification of some samples resulted from poor quality genomic DNA, most likely a result of the poor preservation of tissue and the highly degraded state of carcasses when samples were taken (see section 2.8). Due to the wide geographic spread and low numbers of samples from some locations around Australia, samples were grouped into larger geographic demes for analysis. For definitions of groupings refer to section 2.1.1. The demes used in this study are Shark Bay (n = 22), North Western Australia (n = 23), Northern Territory (n = 4), Torres Strait (n = 131), Northern Queensland (n = 32), Central Queensland (n = 46), and Southern Queensland (n = 87). The samples from outside Australia were grouped as Asian samples (n = 23) and Pacific samples (n = 4).

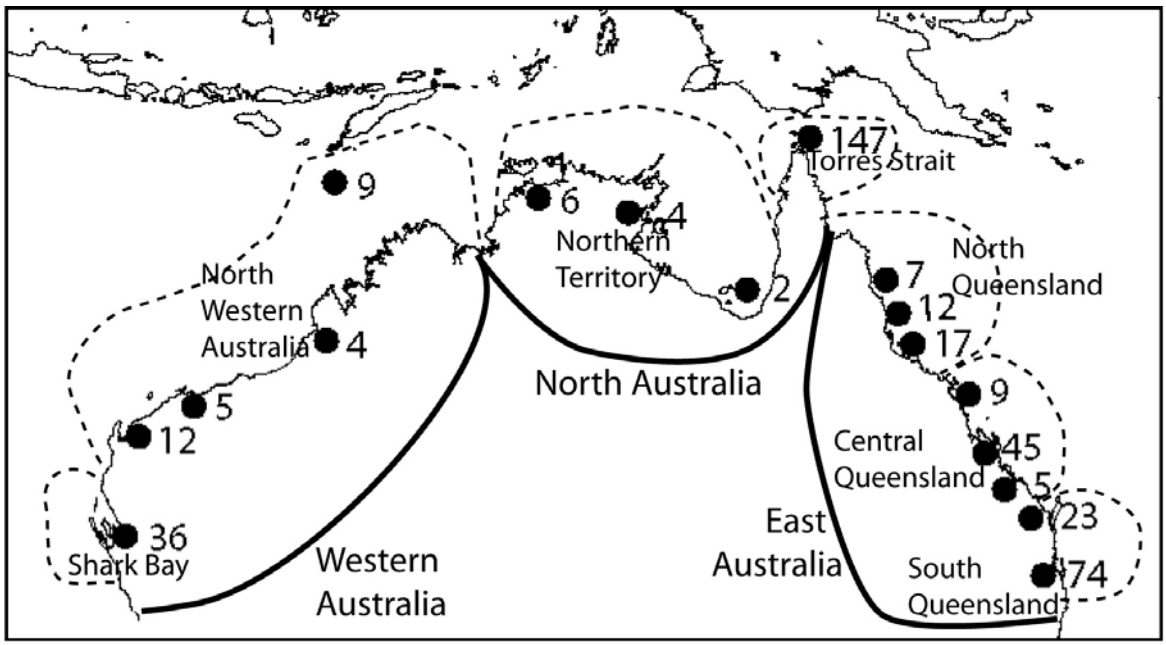



**Figure 5.1** Map indicating the populations of the 417 dugong samples around Australia, and the number from each. The dotted line indicates the grouping of populations into demes for analyses. Grouping of demes into regions is shown by the solid line.

### *5.2.2 Microsatellite loci*

Numerous attempts were made at developing dugong-specific microsatellite loci. Although several microsatellites were found, the limited flanking regions did not allow appropriate primers to be designed. Therefore microsatellites that had been previously developed for the Florida manatee were employed in this study. Initially, nine microsatellite loci isolated from the Florida manatee by Garcia-Rodriguez *et al.* (2000) were tested for variability in 16 dugong samples. Seven of the nine were found to be variable, amplified consistently and were easy to score reliably. These seven were then used to screen the remaining dugong samples. Locus TmaA09 failed to amplify many of the samples and was therefore removed from the analyses. Six loci (TmaA01, TmaA02, TmaA04, TmaE08, TmaE26, and TmaM79) were used in all analyses. Loci TmaA01, TmaA04, TmaE08, and TmaE26 are interrupted compound repeats, locus TmaA02 is a compound di-nucleotide repeat and TmaM79 is a simple di-nucleotide repeat (Table 5.1).

**Table 5.1** Microsatellite loci used in this study isolated in the Florida manatee by Garcia-Rodriguez *et al.* (2000). Repeat type and primer sequences taken from Garcia-Rodriguez *et al.* (2000). The allele size range and allele number ($N_a$) identified in the dugong (this study) are given.

| Locus | Size | Repeat type and length | $N_a$ | Primer sequence |
|---|---|---|---|---|
| TmaA01 | 107 - 113 | $(TA)_3(CA)_3CG(CA)_7$ | 6 | F - CAGAAGGGATACATATACA<br>R - CAGCCCCTGGCTGTCTCTTGTC |
| TmaA02 | 236 - 257 | $(CACT)_2(CA)_{16}$ | 25 | F - CTCAGTCCAAACAGCTAATG<br>R - TAGTCATTTGTGCAGAGTGC |
| TmaA04 | 199 - 225 | $(CT)_2(GT)_{12}AT(GT)_7AT(GT)_2$ | 27 | F - GAACACAAGACCGCAATAAC<br>R - TGGTGTATCACTCAGGGTTC |
| TmaE08 | 168 - 186 | $(CA)_{13}TA(CA)_5$ | 8 | F - GAATAGAGACTGGGCTAGAATCC<br>R - GCCTTTTGGAGGGATAGAAGTAG |
| TmaE26 | 170 - 194 | $(CA)_8C(CA)_{17}$ | 17 | F - CATTCCTGATCCACAAAATC<br>R - CCTGTCTTCTCTCTGTTTCTCC |
| TmaM79 | 151 - 171 | $(GT)_{15}$ | 18 | F - CCAATCATGTCCCAAACT<br>R - CAATAGAAGAAGCAGCAG |

### *5.2.3 PCR amplification and automated scoring*

Each 10 µL PCR reaction contained 10 ng of genomic DNA, 1 x PCR buffer (containing tris-HCl, KCl, and $(NH_4)_2SO_4$), 0.09 mM of dNTPs, 0.45 U of *Taq* DNA polymerase and 0.45 µM of each primer. One of each primer pair (Table 5.1) was

labelled with proprietary fluorescent tags (HEX, TET or FAM) to allow viewing of fragment lengths in an automated fragment analyser. A touch-down PCR was used as follows: denaturing step at 94°C for 30 s, followed by a primer-annealing step for 30 s and a polymerisation step at 72°C for 30 s. For all microsatellites the initial annealing temperature was 60°C, in each consecutive cycle the annealing temperature was reduced by 1°C until the final annealing temperature of 52°C was reached. An additional 28 cycles of the denaturing, annealing and polymerisation were conducted when the final annealing temperature was reached. Products were run on a MegaBace 1000 Genetic Analyzer (Amersham Bioscience) using ET 400-Rox (Amersham Bioscience™) internal standard. Allele sizes were then determined using Fragment Profiler v1.2 (Amersham Bioscience™).

### *5.2.4 Statistical analyses*

The frequency of alleles, observed and expected heterozygosity, and fixation index were determined for each locus and for each population around Australia, using GenAlEx V.6 (Peakall and Smouse, 2005). A deme pairwise matrix of Nei's genetic distance was also calculated in GenAlEx V.6 (Peakall and Smouse, 2005). All loci were tested for Hardy-Weinberg equilibrium and linkage disequilibrium for each deme and region using GenePop (Raymond and Rousset, 1995). BOTTLENECK v1.2.02 (Piry *et al.*, 1999) with 1 000 replications was used to test for the signature of a bottleneck within each deme.

The matrix of deme pairwise Nei's genetic distance was used, along with a matrix of the geographic distances (see section 4.2.8, for assessment method), to test for isolation-by-distance using IBD (Bohonak, 2002). To visualise the differences between demes a neighbour joining tree of the deme pairwise Nei's genetic distances was constructed in PAUP V.4.10b (Swofford, 2000).

Population differentiation was tested using AMOVA (Excoffier *et al.*, 1992) in GenAlEx (Peakall and Smouse, 2005) at a number of hierarchical groupings. The first was between Australian dugongs and the samples from outside Australia. The second

was three regions around Australia (Western Australia, Northern Australia, and Eastern Australia). The third level was between the seven demes in Australia (Figure 5.1). To try and elucidate any distinct population structure present in the data, STRUCTURE V.2 (Falush *et al.*, 2003) was used, with models run for 1-10 populations. Conditions for running STRUCTURE included a burn-in of 10 000 replicates, with 50 000 MCMC replicates, the admixture model with correlated allele frequencies between populations. In total, 10 iterations for each predicted number of populations (K) were performed. Additional exploration of the output from STRUCTURE were conducted following Evanno *et al.* (2005).

## 5.3 Results

### *5.3.1 The utility of Florida manatee microsatellites in the dugong*

High allelic diversity was identified in the dugong samples examined (Table 5.2). The diversity is greater than that found in the Florida manatee (Table 5.2). For example, at locus TmaA02, 25 alleles were identified in the 372 dugong samples, with only three alleles being found in the 223 Florida manatee samples (Table 5.2; Garcia-Rodriguez *et al.*, 2000). Although many alleles were identified for the dugong there is a low number of very common alleles and a high number of rare alleles. This result is observed in the distribution of alleles among the demes (Appendix 7). The effective number of alleles ($N_a = 1 / 1 - H_e$) is consistently lower than the number of alleles observed in all regional categories (Figure 5.2), indicative of high homozygosity across all loci.

**Table 5.2** Allelic diversity, as indicated by the number of alleles in dugongs compared with the manatee (data from Garcia-Rodriguez 2000; Garcia-Rodriguez *et al.* 2000). Dugong (*Dugong dugon*); Florida manatee (*Trichechus manatus latirostris*); Antillean manatee (*T. m. manatus*); Amazonian manatee (*T. inunguis*); n = number of samples.

| ***Species*** | ***Microsatellite Locus*** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **n** | **TmaA01** | **TmaAo2** | **TmaA04** | **TmaE08** | **TmaE26** | **TmaM79** |
| Dugong | 372 | 6 | 25 | 27 | 8 | 17 | 18 |
| Florida manatee | 223 | 1 | 3 | 1 | 3 | 2 | 3 |
| Antillean manatee | 21 | 2 | 3 | 3 | 4 | 8 | 3 |
| Amazonian manatee | 7 | 2 | 5 | 1 | 2 | 1 | 3 |

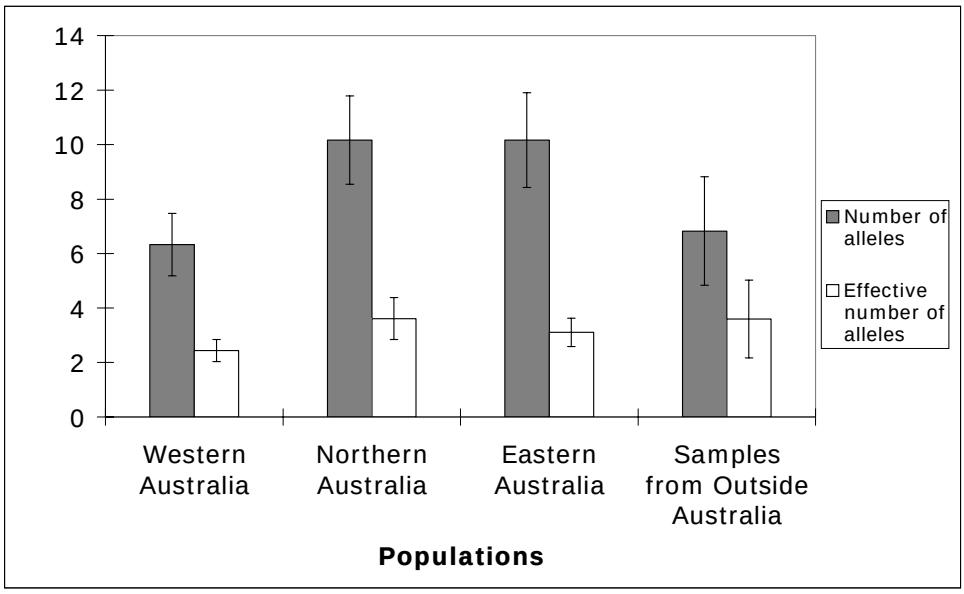



**Figure 5.2** The number of observed alleles and effective number of alleles calculated within regions for six microsatellite loci in the dugong (± SE).

No locus is in Hardy-Weinberg equilibrium (HWE) at the 0.05 level of significance if the whole dataset is considered one population. To avoid the Wahlund effect (Wahlund, 1928) the data set was separated into the demes (shown on Figure 5.1) and tested to see if any conformed with HWE. When this was done, several loci still did not conform to HWE, although these loci differed across demes (Table 5.3). With the exception of locus TmaA01, the only region that consistently did not conform to HWE contained the samples collected from outside Australian waters (Table 5.3). The loci were also tested for linkage disequilibrium and results indicated that all pairs of loci were in equilibrium. The fixation index ($F = (H_e - H_o) / H_e$) was approximately 20% across all loci. This result indicates that inbreeding within the dugong is occurring at a low but possibly important level.

**Table 5.3** Probability outcomes from tests of six microsatellite loci for conformation to the Hardy-Weinberg equilibrium across the different demes. Significant values at the 0.05 level are in bold italics. '-' indicates locus was monomorphic for this deme.

| | **TmaA01** | **TmaA02** | **TmaA04** | **TmaE08** | **TmaE26** | **TmaM79** |
|---|---|---|---|---|---|---|
| **Shark Bay** | 0.19 | ***0.01*** | 0.78 | 1 | ***0.03*** | 0.22 |
| **North WA** | 0.56 | 0.12 | ***0.02*** | 1 | ***0.03*** | ***0.02*** |
| **Northern Territory** | 1 | 0.22 | 1 | - | 1 | - |
| **Torres Strait** | ***0.00*** | ***0.00*** | 0.24 | *0.04* | ***0.00*** | 0.57 |
| **North Queensland** | 0.70 | 0.06 | 0.45 | 0.57 | 0.05 | 0.07 |
| **Central Queensland** | ***0.03*** | 0.09 | 0.25 | 0.94 | 0.23 | ***0.00*** |
| **South Queensland** | ***0.00*** | 0.13 | 0.09 | 0.10 | ***0.00*** | ***0.00*** |
| **Outside Australia** | 1 | ***0.00*** | ***0.00*** | ***0.00*** | ***0.02*** | ***0.00*** |

Although the loci do not conform with HWE the microsatellite loci are shown to be inherited co-dominantly. A total of 18 mother and calf pairs were collected and screened with these 6 microsatellite loci. Of these, eight mother-calf pairs amplified at 50% or more of the loci. These pairs indicate that the all loci are inherited bi-parentally with no evidence of null alleles appearing in the data set (Table 5.4). Interestingly, one mother-calf pair had the same genotype. This is not surprising given that I identified a low number of very common alleles and a deficit of heterozygotes in the population.

**Table 5.4** Multilocus genotypes of nine mother-calf pairs indicating the co-dominant inheritance of the six microsatellites used in this study. '-' indicates missing data. Possible paternal alleles are boxed.

| | **Locus** | | **Locus** | | **Locus** | | **Locus** | | **Locus** | | **Locus** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **TmaEo8** | | **TmaAo4** | | **TmaM79** | | **TmaE26** | | **TmaAo1** | | **TmaAo2** | |
| Mother | 172 | 174 | 201 | 215 | 163 | 167 | - | - | 109 | 111 | 238 | 240 |
| Calf | 172 | 174 | 201 | 215 | 163 | 167 | 182 | 182 | 109 | 111 | 238 | 240 |
| Mother | 170 | 174 | - | - | 163 | 167 | 180 | 182 | 109 | 109 | 238 | 250 |
| Calf | 184 | 174 | 209 | 215 | 163 | 167 | 180 | 182 | 109 | 109 | 238 | 250 |
| Mother | 172 | 184 | 221 | 221 | 163 | 163 | 180 | 182 | 109 | 111 | 236 | 250 |
| Calf | 172 | 172 | 203 | 221 | 159 | 163 | 180 | 174 | - | - | 250 | 250 |
| Mother | 172 | 174 | 221 | 221 | 163 | 163 | 180 | 182 | 109 | 111 | 238 | 238 |
| Calf | 172 | 174 | 221 | 221 | 163 | 163 | - | - | 109 | 109 | 238 | 252 |
| Mother | 172 | 174 | 201 | 201 | 165 | 165 | 180 | 182 | 111 | 111 | 238 | 250 |
| Calf | 172 | 172 | 201 | 221 | 163 | 165 | 182 | 182 | 109 | 111 | 240 | 250 |
| Mother | 172 | 184 | 221 | 223 | 163 | 163 | - | - | 109 | 109 | 250 | 250 |
| Calf | 172 | 184 | 223 | 223 | 163 | 163 | - | - | 109 | 109 | 250 | 250 |
| Mother | 172 | 172 | 201 | 221 | - | - | 182 | 182 | 111 | 111 | 238 | 240 |
| Calf | 172 | 174 | 201 | 209 | - | - | 180 | 182 | 95 | 111 | 240 | 240 |
| Mother | 172 | 184 | 209 | 221 | 163 | 163 | 180 | 182 | 111 | 111 | 238 | 250 |
| Calf | 172 | 184 | 221 | 221 | 163 | 163 | - | - | 95 | 111 | 240 | 250 |

### *5.3.2 Identification of no historical bottleneck*

The results from BOTTLENECK do not suggest that any of the demes around Australia have undergone a recent decrease in effective population size, as the allele frequencies of all demes have a normal L-shaped distribution, and non-significant results in the Wilcoxon test.

### *5.3.3 Isolation-by-distance*

A Mantel test for isolation-by-distance based on Nei's genetic pairwise distance between demes and linear geographic distance showed a significant positive relationship ($r = 0.7427$, $p < 0.001$; Figure 5.3). The neighbour joining tree of Nei's genetic distance between populations shows the broad scale pattern of isolation-by-distance around Australia, and distinctiveness of the Pacific samples (Figure 5.4). Not surprisingly the samples from Asia appear to be most closely related to samples from Torres Strait and the Northern Territory (Figure 5.4).

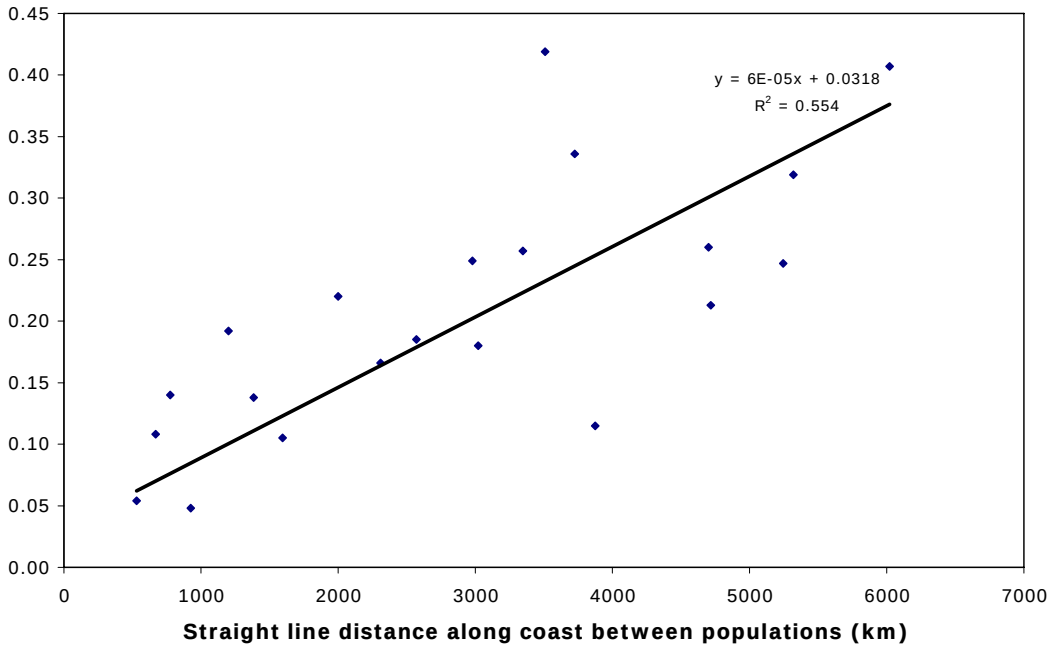



**Figure 5.3** Scatter-plot of the relationship between the population pairwise Nei's genetic distance and linear geographic distances among Australian dugong populations.

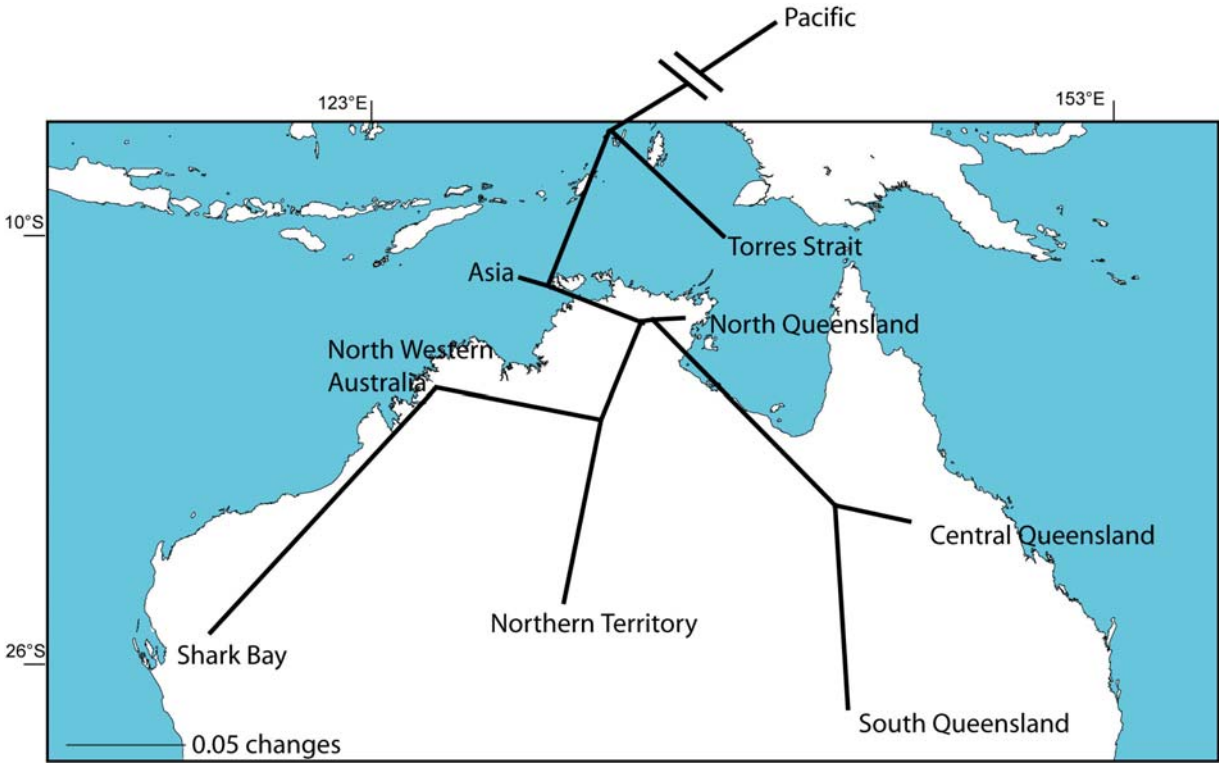



**Figure 5.4** Neighbour joining tree of Nei's genetic distance overlaid on map of Australia to show the landscape scale of isolation-by-distance. Broken line indicates 0.425 changes between Pacific samples and those from Australian waters.

### *5.3.4 Population differentiation*

As summarised in Table 5.5, all AMOVAs produced significant results, except for comparisons between Australia and Overseas populations (Test 2, Table 5.5) so panmixia is rejected at all other scales. All tests also show that most variation occurs among individuals within populations or demes (Table 5.5). Test one indicates that globally dugongs are not panmictic, with 11% variation occurring among populations. Test two showed only 1% variation between the regions (Australia and Outside Australia) and only 11% of the variation among demes within these regions (Table 5.5). Nearly all the variation (88%) was among individuals within demes (Test 2, Table 5.5). However, if we split Australia up into three regions (Western Australia, North Australia and East Australia) and compare these regions with the overseas region, there is a greater amount of variation between regions (7% compared with 1% - Tests 3 and 2 respectively) and a lower amount of gene flow than that indicated in test 2 (Table 5.5). Within Australia, the majority of the variation occurs between individuals within population or deme (Tests 4, 5 and 6, Table 5.4). There is a relatively high level of gene

flow among demes (Test 5), among regions (Test 6), and among demes within regions (Test 6, Table 5.5).

**Table 5.5** Analysis of Molecular Variance Analyses (AMOVA) indicating the amount of variation attributable to each hierarchical grouping (populations within demes within regions), the relevant statistics and significant levels. Geographical localities of populations and deme groupings are indicated in Figure 5.1. Refer to section 2.1.1 for definitions of groupings.

| Test | Hierarchical groupings | % Variation | $\phi$ Statistics | Significance |
|---|---|---|---|---|
| **1** | All populations as one region | 11% among populations<br>89% within populations | $\phi_{st} = 0.113$ | P = 0.01 |
| **2** | All demes as two regions (Australia and Overseas) | 1% among regions<br>11% among deme/region<br>88% individual/deme | $\phi_{rt} = 0.007$<br>$\phi_{sr} = 0.112$<br>$\phi_{st} = 0.118$ | P = 0.13<br>P = 0.01<br>P = 0.01 |
| **3** | Nine demes as four regions (WA, NA, EA, Overseas) | 7% among regions<br>5% among deme/region<br>88% individual/deme | $\phi_{rt} = 0.074$<br>$\phi_{sr} = 0.054$<br>$\phi_{st} = 0.124$ | P = 0.01<br>P = 0.01<br>P = 0.01 |
| **4** | All populations in Australia as one region | 13% among populations<br>87% within populations | $\phi_{st} = 0.129$ | P = 0.01 |
| **5** | All populations in Australia grouped into the seven demes | 6% among demes<br>7% among populations/deme<br>87% individual/populations | $\phi_{rt} = 0.063$<br>$\phi_{sr} = 0.073$<br>$\phi_{st} = 0.132$ | P = 0.01<br>P = 0.01<br>P = 0.01 |
| **6** | All demes in Australia as three regions (WA, NA, and EA) | 8% among regions<br>4% among deme/region<br>87% individual/deme | $\phi_{rt} = 0.082$<br>$\phi_{sr} = 0.048$<br>$\phi_{st} = 0.126$ | P = 0.01<br>P = 0.01<br>P = 0.01 |

As my grouping of demes was arbitrary, the computer program STRUCTURE was used to try to elucidate any subtle population genetic structure. STRUCTURE assesses the log likelihood of the probability that each sample belongs to a "cluster or population" with the number of clusters specified *a priori* for each run. The overall results from STRUCTURE are visualised as bar plots, where each cluster or population is given a colour (Figure 5.5). If there is distinct structuring, blocks of colour should appear when samples have a high probability of occurring in a particular cluster or population. As illustrated (Figure 5.5) there appears to be no significant geographic structure with only some samples from Shark Bay forming a distinct and unique cluster when more than two populations are specified. As the number of populations specified increases from two (Figure 5.5A) to nine (Figure 5.5H) the pattern appears progressively worse. The

most appropriate number of populations based on the output appears to be two or three. (Figure 5.5A or 5.5B). The pattern identified when two clusters are specified (Figure 5.5A) appears to be that indicative of isolation-by-distance that has already been identified in the data (section 5.3.3; Figure 5.3). In order to identify the true number of populations and interpret the STRUCTURE results more rigorously a number of graphs based on the estimate of log likelihood of the probability of the data (LnP(D)) for a given number of populations were produced following Evanno *et al.* (2005). These calculations failed to identify a 'true' number of populations indicating a lack of geographic structuring of dugongs around Australia (Figure 5.6). The rate of change of the likelihood distribution (graph B, Figure 5.6) should decrease when the true number of populations is reached, this result does not apply to dugongs. Similarly, there is no peak in the absolute values of the second order rate of change of the likelihood distribution (graph C, Figure 5.6) at the true number of populations. The distribution of $\Delta$ K (graph D, Figure 5.6) should produce a peak at the true number of populations. The peak produced in the dugong data is at eight populations, which has a high amount of error associated with it as indicated in graphs A to C (Figure 5.6). There is a small peak at two populations, but the delta K values are very low, suggesting that this is not the true number of populations (graph D, Figure 5.6).

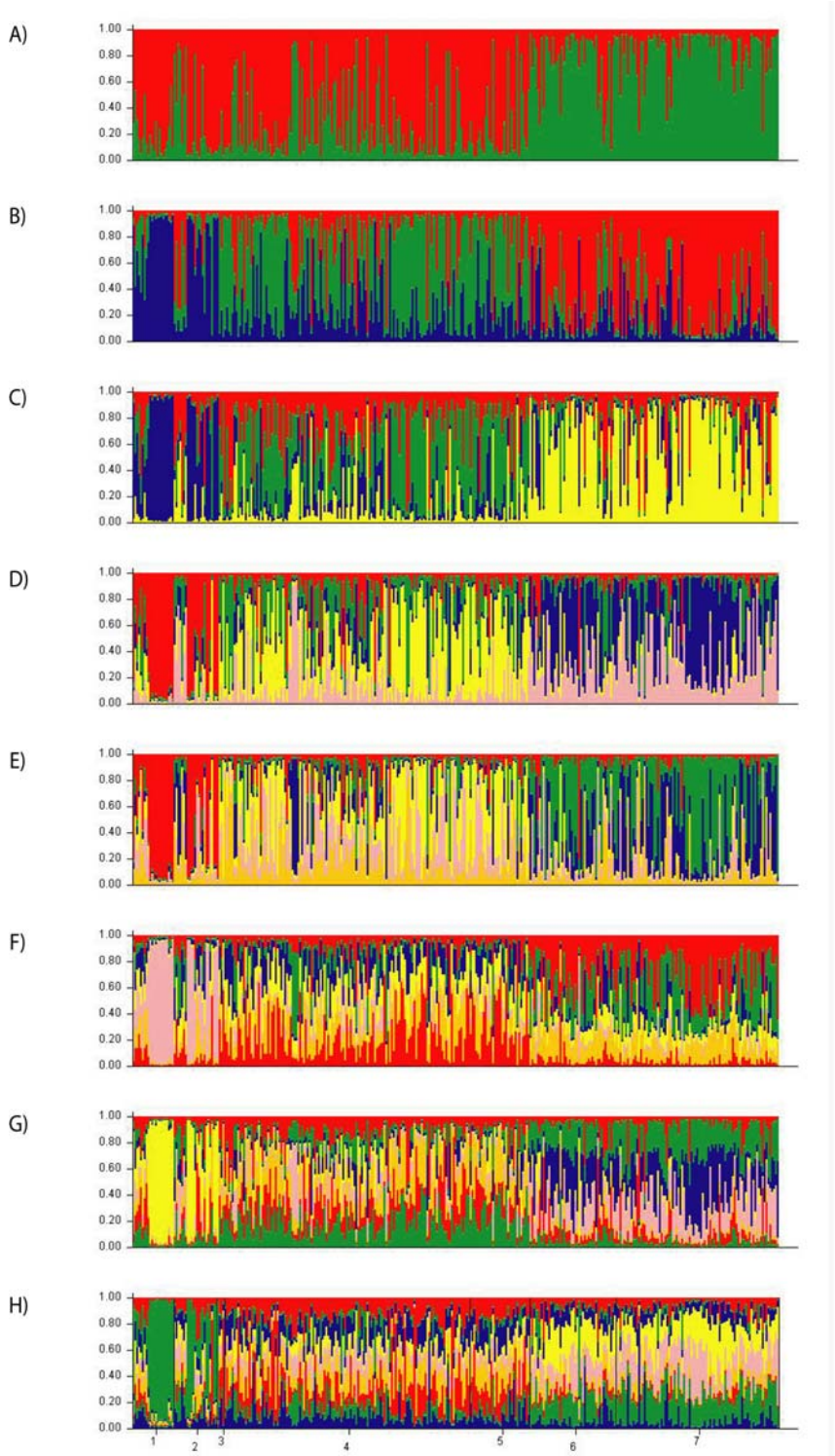



**Figure 5.5** Bar plots outputs from STRUCTURE. Each column (vertical line) within the plot is one individual. Each colour represents a different cluster or population and the proportion of a single colour in a column represents the probability of that sample belonging to that population. All graphs are sorted by geographic deme. 1 = Shark Bay; 2 = North Western Australia; 3 = Northern Territory; 4 = Torres Strait; 5 = North Queensland; 6 = Central Queensland; 7 = South Queensland. A) Number of populations (K) = 2; B) K = 3; C) K = 4; D) K = 5; E) K = 6; F) K = 7; G) K = 8; H) K = 9.

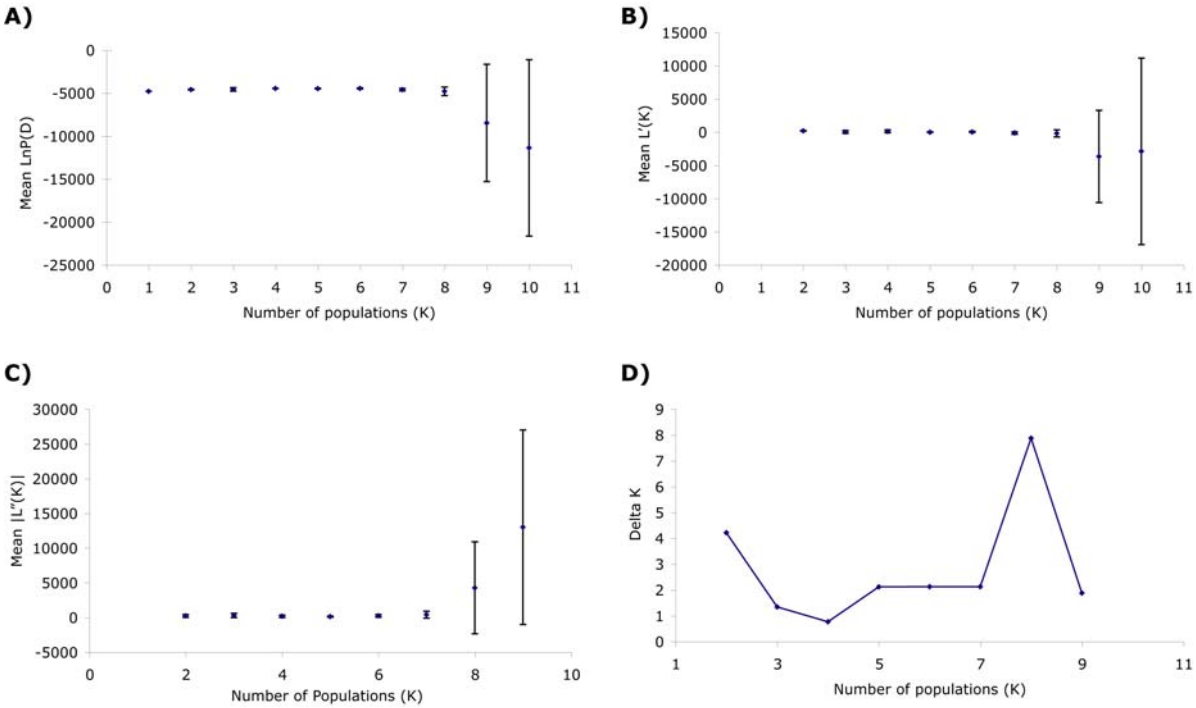



**Figure 5.6** Interpretation of the output from structure following Evanno *et al.* (2005) to identify the true number of populations within dugongs around Australia. A) The mean estimate of the log likelihood of the probability of the data (LnP(D)) (mean ± SE). B) Rate of change of the likelihood distribution (mean ± SE). C) Absolute values of the second order rate of change of the likelihood distribution (mean ± SE). D) Δ K. The modal value of this distribution is the true number of populations or the uppermost level of structure. For dugongs the peak at two populations is a low Δ K value and the peak at eight populations is in part of the distribution with high standard errors, therefore one population of dugongs occur around Australia.

## 5.4 Discussion

### *5.4.1 High variability of microsatellites reflect the lack of a population bottleneck*

The greater number of alleles observed in the dugong compared with the Florida manatee is unusual as the microsatellite loci used in this study were developed for the latter species. Although the dugong and manatee are sister taxa, they occur in different families (Dugongidae and Trichechidae) and last shared a common ancestor millions of years ago. Garcia-Rodriguez *et al.* (2000) suggest the low genetic diversity and variability in the Florida manatee is an indication of a founder effect or major population bottleneck of evolutionary significance. We can then assume the high genetic diversity in the dugong suggests that it has not undergone a severe bottleneck.

This conclusion is supported by results from BOTTLENECK v1.2.02 (section 5.3.2). The evidence that the dugong has not undergone a population bottleneck does not contradict coalescent analyses conducted in chapter 4 (section 4.3.6), which indicate a historical population expansion. Although the dugong population has undergone a historical expansion, the population must have remained relatively stable afterwards, as there is no signature in the microsatellite data, which examines a more contemporary time scale compared with the mtDNA data, to indicate a population decline or bottleneck. There is a possibility the dugong has experienced a bottleneck within the last few generations due to anthropogenic causes, but as dugongs are long lived and have a long generation time the genetic effects of this reduction cannot yet be detected.

### *5.4.2 Lack of Hardy-Weinberg equilibrium reflects non-random mating*

Although it is common for some loci or isolated populations to deviate from Hardy-Weinberg equilibrium (HWE) it is unusual that all loci over all populations fail to conform to HWE as is the case for the dugong. As HWE is the basic assumption of many genetic analyses, it is essential to consider the reason underlying the disequilibrium of the microsatellites in dugongs. In order for loci to fulfil HWE a number of assumptions need to be met. These assumptions are listed in section 2.4.1. Calculation of HWE assumes the organism in question is diploid and reproduction is sexual. Both of these assumptions are met and well documented for dugongs (Preen, 1989; Anderson, 1997; Anderson, 2002). A third assumption of HWE is that the population will reach equilibrium in one generation if the organism in question has generation times that are not overlapping. This assumption is violated in dugongs as the generation time (average age of adults in the population) is approximately 27 years (H. Marsh pers. com. 2005), which means generations will overlap in the population. The result of overlapping generations is that the population will take longer to reach equilibrium than one generation. Although this assumption of HWE is not met, it is unlikely to be the reason for the Hardy-Weinberg disequilibrium seen in dugongs as they have probably been in Australian waters for approximately three million years. This date coincidences with the beginning of the Pleistocene – the era to which dugong fossil fragments found in the Indo-Pacific belong (Domning and Furusawa, 1995; Domning, 2001). Three MY is approximately 111 111 generations and should be long enough for equilibrium to be reached. Even more recent changes in population genetic

diversity would be expected to have reached equilibrium, given the overall genetic diversity observed within the microsatellite data, and the lack of any signature of bottlenecks in the data.

The model of HWE assumes if the population numbers are large then the effects of migration, mutation and natural selection are negligible. The number of dugongs around Australia today is estimated to be 100 000 based on aerial survey data (H. Marsh pers. com. 2005). The historical population sizes calculated from the mtDNA data (section 4.3.6) indicate that the dugong populations were larger in the past. This present large population size means that the effects of migration, mutation and natural selection are neglible, and therefore can be dismissed as the reason for the observed disequilibrium.

Random mating is also a requirement of HWE. Several behavioural observations suggest mating may not be random within dugong populations. Dugongs on Australia's east coast have a promiscuous mating system with the female being surrounded by a number of males (Preen, 1989). This behaviour, in theory, should be a form of random mating as each of the males surrounding the female is just as likely to mate with her. However, if only the strongest male in a population succeeds in fertilising the female this behaviour may constitute non-random mating, as a few dominant males may potentially mate with all the females in the area. On the west coast of Australia, males are believed to defend territories called 'leks' in the hope of attracting a female (Anderson, 1997; Anderson, 2002). This behaviour is also likely to result in a non-random mating system. The importance of this 'lek' behavioural system is unknown at present, it is possible that it only occurs sporadically and may not be the primary mating mechanism for dugongs on the west coast of Australia. Observations of dugong mating systems are hard to validate as the sex of a dugong cannot be determined without intervention.

Dugongs also have an asynchronous, discontinuous breeding season (Marsh *et al.*, 1984), which is diffusely seasonal in the tropics. Males appear to be in breeding condition in a temporally staggered way with no sharply defined male breeding season (Marsh *et al.*, 1984). Individual male dugongs are not in continuous breeding condition

(Marsh *et al.*, 1984). Female dugongs appear to be in breeding condition during the second half of the year (Marsh *et al.*, 1984). This lack of a definite breeding season and the asynchronicity of breeding condition may contribute to a non-random mating system.

Other factors that may result in non-random breeding, include inbreeding and dissortive mating (actively avoiding inbreeding). Inbreeding is the mating of individuals that are more closely related than average pairs in the population. The fixation indexes of the microsatellite data obtained in this study indicate that inbreeding is occurring at approximately the 20% level. Therefore, inbreeding cannot be discounted as contributing to the reason the loci do not conform to HWE. However the high level of diversity within the microsatellites contradicts the possibility that dugongs are actively undergoing inbreeding, unless small family groups are inbreeding. There is no evidence for strong social structure or family groups being present in the dugong, with the exception of the mother-calf bond (Anderson, 1981). However, there is insufficient research conducted on this aspect of dugong ecology at present to discuss this conclusion at greater depth. Dissortive mating is when animals actively avoid inbreeding. When this occurs there is a higher number of heterozygotes in the population than expected. Within my dataset there is a lower than expected observed heterozygosity, as indicated by the low number of effective alleles (section 5.3.1). I therefore dismiss dissortive mating as the reason for loci not conforming to HWE expectations. This biological and genetic information on the behaviour of dugongs supports the possibility that non-random mating may be occurring in the dugong populations around Australia and warrants further investigation.

Other possible reasons for the lack of HWE within the data set include: the Wahlund effect, presence of null alleles, and fragmented populations with restricted gene flow. The Wahlund effect is a sampling artefact. If a series of small non-interbreeding populations are sampled and analysed under the false assumption that they all belong to the same population then the populations are more likely to be out of HWE. The fact that loci did not conform to HWE expectations when tested at a number of smaller sampling groupings, and the lack of strong social structure in the dugong (see above), dismisses the Wahlund effect as the reason for the disequilibrium. The presence of null

alleles is possible as they are more common when cross-species amplification is used as it was here. When looking at the mother-calf pairs, no null alleles were detected. Therefore, the possibility of a high frequency of null alleles within the data set is low. None of the analyses conducted suggests that dugongs have a fragmented population with restricted gene flow, therefore this explanation for data not conforming to the HWE can also be excluded.

The most likely reason for the lack of HWE is non-random mating occurring either due to the differing breeding systems around Australia or the asynchronous breeding season. However, a combination of low-frequency null alleles and a low level of inbreeding that may produce a significant variation from the HWE, cannot be ruled out. Interestingly Garcia-Rodriguez (2000), also reported for the Florida manatee that some microsatellite loci were not in HWE in some populations and contained an excess of homozygotes. Garcia-Rodriguez (2000) came to the conclusion that this result was most likely to be the caused by inbreeding and the possibility of null alleles within the dataset. Interestingly, the Florida manatee has a breeding system like that of dugongs on the east coast of Australia (Rathbun *et al.*, 1995).

### *5.4.3 Lack of geographic structuring reflects a high level of gene flow and migration*

The analyses conducted failed to uncover any significant geographic population structuring across Australia. The only differentiation detected is isolation-by-distance. This indicates that there is connectivity between adjacent localities; the genetic distance increases with increase in geographic distance. This isolation-by-distance effect is probably responsible for the significant $\phi$ values produced in the AMOVA (Table 5.5). Isolation-by-distance is not a surprising finding given the spatial scale over which samples were obtained. There were no significant groupings within the STRUCTURE analyses. Similarly the AMOVA results indicated that notional regions (Western Australia, North Australia, and East Australia) are still highly connected with only 8% of the total variation occurring among the regions and a high level of gene flow among regions as indicated by a $\phi_{rt}$ of 0.082.

Because of this isolation-by-distance the dugong population cannot be called a panmictic population, where every individual has an equal opportunity to breed with each other. There are limits to the connectivity of the dugong populations, for example, dugongs in Shark Bay (on the west coast of Australia) are unlikely to travel to Moreton Bay (on the east coast of Australia), a distance of approximately 6000 km, to breed. By definition it is impossible to have a panmictic population of a species that spans a large geographical area unless they all migrate to a specific area for breeding. For dugongs, there is no indication of specific breeding area (except perhaps so called lekking site in Shark Bay (Anderson, 1997), the significance of which is not known).

If populations are not panmictic, they are normally considered a metapopulation that is a series of interconnected subpopulations (see Chapter 2.1.1 for definition). It is not appropriate to classify the dugong as a metapopulation as distinct subpopulations could not be identified (see section 5.3.4). However, not all organisms that show isolation-by-distance have been classified as a metapopulation. A study by Anderson *et al.* (2004) indicated that cougars in the Wyoming Basin display isolation-by-distance. However, these authors preferred to consider the cougars as one population, not a metapopulation. Similarly, the label of a metapopulation is difficult to apply to dugongs because of the high variability of individual movements and lack of definite, static, subpopulations. The individualistic movements of dugongs and the varying size of their 'home ranges' [0.5 $km^2$ to 733 $km^2$ (Sheppard *et al.*, in press)] make it difficult to delimit geographically isolated subpopulations. Furthermore, the high amount of genetic connectivity between geographic regions suggest that the dugong does not fit a traditional metapopulation structure. My data, interpreted in the light of the dugongs' ecology and behaviour, indicate that dugongs need to be studied and managed at the landscape scale (see Chapter 7). This conclusion is especially important considering the connectedness of bays and seagrass meadows as previous movement data and results from this study indicate.

This study and the lack of strong geographic structuring is similar to findings of past studies on sirenians and contrasts previous studies on many marine mammals. A study on the Florida manatee by Garcia-Rodriguez (2000) identified shallow genetic differentiation between the east and west coasts of the Florida Peninsula. However,

there was a high amount of gene flow between regions on each coast and some gene flow between coasts (Garcia-Rodriguez, 2000). Other studies on marine mammals using microsatellites have shown some population differentiation at large scales (see section 2.7), which is not evident in the dugong with non-significant AMOVA results between Australia and samples from outside Australia (Test 2, Table 5.5). One reason for the differences in results between sirenians and other marine mammals is the necessity for sirenians, especially dugongs, to remain relatively close to the coast and the seagrass meadows on which they forage. Another reason for the difference between dugongs and other marine mammals is the lack of specific breeding/calving grounds [with the exception of the lekking site in Shark Bay (Anderson 1997)] and the probable lack of any strong social structure within the dugong (Anderson, 1981).

What is clear from this study is that dugongs that move are likely to mate as is indicated by the lack of geographic structuring. Dugongs rely on a highly variable (both temporally and spatially) resource, seagrass. This means that dugongs undergo adaptive movement to ensure survival (Gales *et al.*, 2004; Marsh *et al.*, 2004). This behaviour is not uncommon for large herbivores. Herbivores on the African plains were found to move adaptively to acquire forage when needed (Fryxell *et al.*, 2005). Similar behaviour has also been identified for kangaroos in arid Australia (Norbury *et al.*, 1994). The dugong is difficult to assign to discrete populations as a result of its adaptive movements and longevity. As there is a complex interaction at a large scale between the matrix of seagrass resources, dugong movement and gene flow indicated in this study, future studies of dugongs must be on a landscape scale.

### *5.4.4 Summary*

The microsatellites developed for the Florida manatee are highly variable in the dugong. These loci have greater allelic diversity in the dugong than in the manatee. The high allelic diversity and the non-significant results in the Wilcoxon tests, conducted in BOTTLENECK v1.2.02 (Piry *et al.*, 1999), suggest the dugong population has not undergone an extensive bottleneck or founder effect. This result contrasts with the results for the Florida manatee, which suggested that the population had undergone an extensive bottleneck (Garcia-Rodriguez *et al.*, 2000). The high allelic diversity within

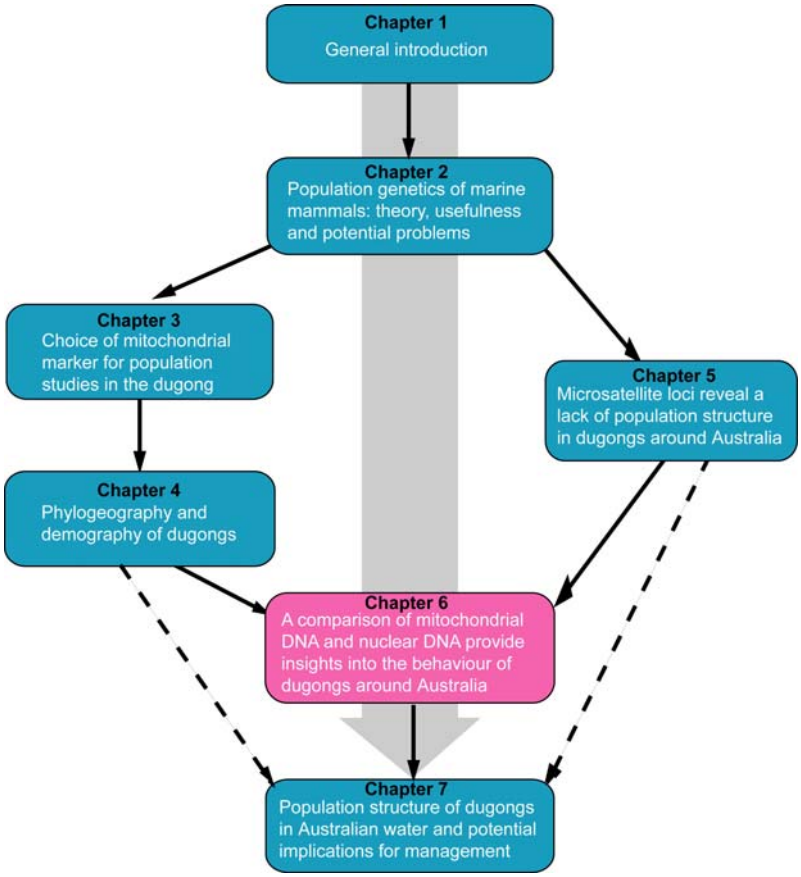
the dugong is also an indication of the larger geographic area and larger population numbers of the dugong compared with the manatee.

The microsatellite loci used do not conform to HWE in dugongs. The most probable cause for this lack of equilibrium is non-random mating that is facilitated by the differing mating systems around Australia and by asynchronous breeding season. Another possibility, which cannot be dismissed, is a combined effect of low-frequency null alleles and inbreeding, resulting in genotype frequencies that are not in HWE.

There is a lack of population differentiation at a large geographic scale for dugongs around Australia and between Australia and overseas. This lack of structure indicates that there is a high level of gene flow and migration occurring. More importantly it indicates that a percentage of dugongs that make long distance journeys are mating at the other end. Isolation-by-distance is observed over the Australian scale, which is not surprising considering the vagility of the dugong and the geographic scale of my sampling. Significantly, there appears to be limited concordance between the mtDNA and microsatellite data. Further analyses of the combined datasets will be undertaken in the next chapter.

# Chapter 6: Comparison of mitochondrial DNA and nuclear DNA markers provide insights into the behaviour of dugongs around Australia

*This chapter compares and contrasts the mitochondrial DNA sequence (control region) and the nuclear DNA (microsatellite) datasets obtained in this study. The mtDNA dataset identified two distinct lineages that have an incomplete geographical overlap around Australia. This pattern is thought to be a result of barriers to gene flow at times of low sea level in the past. The nuclear DNA dataset failed to identify any population structuring other than isolation-by-distance at the Australian scale. This result indicates gene flow and movement between neighbouring locations and perhaps over large spatial scales. The apparent lack of concordance between the two datasets suggests interbreeding between the two mitochondrial lineages and the potential for sex-biased dispersal.*



## 6.1 Introduction

In genetic studies of natural populations, the importance of using multiple loci to obtain a better understanding of the processes responsible for observed patterns has been noted (Chapter 2). It is especially useful to compare results from loci with different inheritance modes such as mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Results from mtDNA and nDNA complement each other by revealing different aspects of the genetic partitioning within the study species, and allow a fuller understanding of ecological and biological processes acting on the population as whole. The mtDNA is commonly used to identify the historical interactions between populations for a number of reasons: 1) high variability, 2) lack of recombination and 3) maternal inheritance (Moritz *et al.*, 1987; Hoelzer *et al.*, 1998). For organisms unlikely to show gender-specific dispersal patterns, the maternal inheritance of a marker may not be an issue. However, for species with complex behaviour, maternal inheritance can be a problem, as such a marker only identifies female-mediated gene flow. In mammals, males tend to disperse further from their birth place and also tend to move greater distances between breeding seasons and sites than females (Greenwood, 1980). With this in mind, it is essential to identify all the gene flow that is occurring within a population of mammals. Therefore nDNA, which is inherited bi-parentally, should also be analysed. By comparing the patterns of gene flow produced from both marker systems, gene flow mediated by males in the population can be indirectly accounted for, and hence the behaviour of the study organism can also be inferred.

This study has used sequence data from the mtDNA control region and six nuclear microsatellite loci to explore the population genetic structure of the dugong in Australia. As expected, the mtDNA sequence data was most useful for investigating the historic and large scale relationships among dugong populations. The mtDNA sequence data clearly differentiated two lineages of dugongs around Australia, one widespread and one that was more restricted in geographical distribution (Chapter 4). The mtDNA also clearly identified a difference between the dugongs from Australia and from Asia (Chapter 4). The markers of choice for nDNA analyses were microsatellite loci. These markers allowed current population genetic structure and processes occurring in the dugong populations to be investigated. The nDNA identified significant gene flow occurring among dugongs, both between Australian and Asian samples, and among

Australian dugongs (Chapter 5). No distinct population structuring was identified (Chapter 5). However, there is a significant level of isolation-by-distance at the Australian scale (Chapter 5). Although interesting and important information was obtained using each dataset, additional insights into the ecology and behaviour of dugongs may be inferred by combining them.

In this chapter I aim to combine information obtained from both mtDNA and nDNA to enhance the understanding of dugong behaviour and ecology. The apparent discrepancies between the observed patterns of the datasets will be explored. More specifically, interactions of the two distinct mtDNA lineages in the zone of geographical overlap will be examined.

## 6.2 Methods

In order to evaluate the specific concordance between the two data sets produced in this study, the microsatellite dataset was reduced to those individuals for whom mtDNA sequences were also obtained (n = 115). The microsatellite dataset was then further reduced by removing samples that failed to amplify for more than 50% of the microsatellite loci (final n = 81). The position of haplotype 34 between the two lineages in Australia makes assigning this haplotype to a lineage difficult. The individual with this haplotype was removed from analyses for this reason. A principal co-ordinates analysis, based on the microsatellite DNA, was conducted in GenAlEx 6 (Peakall and Smouse, 2005) on the pairwise genetic distance between individuals identified by the mitochondrial lineage to which they belong. An AMOVA was conducted on the microsatellite data in GenAlEx 6 (Peakall and Smouse, 2005), with the assigned mitochondrial lineages as the populations.

The population pairwise $F_{st}$ and Nei's genetic distance for each lineage based on the microsatellite data were calculated in GenAlEx 6 (Peakall and Smouse, 2005). Population pairwise $F_{st}$ and uncorrected 'p' genetic distances for the mitochondrial sequence data were calculated in ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000). To identify any associations between the two datasets for the widespread lineage, a Mantel test on the matrices of population pairwise $F_{st}$ was conducted in IBD (Bohonak, 2002).

Due to the high number of negative $F_{st}$s obtained for the mtDNA of the restricted lineage, a Mantel test was also conducted on the two population pairwise genetic distances matrices (Nei's and pairwise differences for the microsatellite and mtDNA respectively) for this lineage in IBD (Bohonak, 2002). Although the measures of genetic distance are different, they are the most comparable distance measures available for the two different data sets.

Although the direct comparison of samples of known mtDNA lineage with the results from nDNA is useful, the reduced sample size weakens the inferences. A summary table of the AMOVA results including the entire data sets from chapters 4 and 5 was produced. Comparison of these results with larger sample sizes will increase the strength of the findings.

## 6.3 Results

A principal co-ordinates analysis conducted on the genetic distance based on microsatellite DNA identified 35% of the variation by the first three axes, with the first axis accounting for 13.4% (Table 6.1). Inspection of Figure 6.1 shows that the mitochondrial lineages failed to separate into identifiable clusters in this analysis. This result suggests no concordance between the two datasets.

**Table 6.1** The percentage of variation that is accountable by each of the first three axes in the principal co-ordinates analysis on the genetic distances of the microsatellite DNA.

| **Axis** | **1** | **2** | **3** |
|---|---|---|---|
| **% Variation** | 13.4 | 11.3 | 10.2 |
| **Cumulative % variation** | 13.4 | 24.7 | 34.9 |

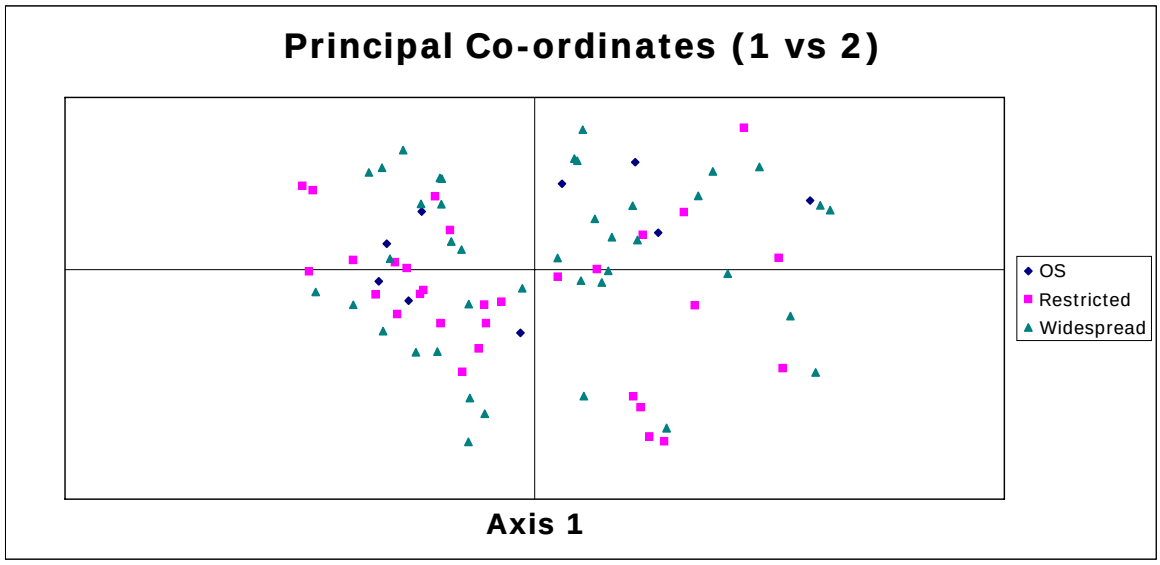



**Figure 6.1** Principal co-ordinates plot of microsatellite DNA pairwise distances for 81 dugong individuals across Australia and overseas separated by mtDNA lineage, the first two axes accounted for 25% of the variation between the lineages.

An AMOVA conducted on the reduced microsatellite dataset indicated that there was only 2% variation between the lineages and 98% variation within the lineages (Table 6.2). This results in a low, although significant, $\phi_{st}$ of 0.02 (p = 0.01) suggesting there is substantial gene flow occurring between the lineages, but not panmixia (Table 6.2). The $\phi_{st}$ value for the microsatellite loci is low in comparison with the $\phi_{st}$ for the mtDNA haplotype data between the lineages ($\phi_{st}$ 0.83; see Chapter 4).

**Table 6.2** AMOVA table based on the microsatellite data with subdivisions being the two Australian lineages identified from mtDNA. df = degrees of freedom, SS = sum of squares, MS = mean square, Est.Var. = estimated variance, % = percentage variation.

| Source | df | SS | MS | Est. Var. | % | | |
|---|---|---|---|---|---|---|---|
| **Between lineages** | 2 | 8.8 | 4.4 | 0.05 | 2% | | |
| **Within lineages** | 157 | 324.2 | 2.1 | 2.06 | 98% | $F_{st}$ | 0.02 |

No Mantel tests found any significant associations between the two matrices produced from the different datasets. The Mantel tests on the widespread lineage were not significant (r = 0.1304, one-sided p <= 0.2850; Figure 6.2). A similar lack of any significant association was found in the restricted lineage (r = 0.0604, one-sided p <= 0.2310; Figure 6.3).

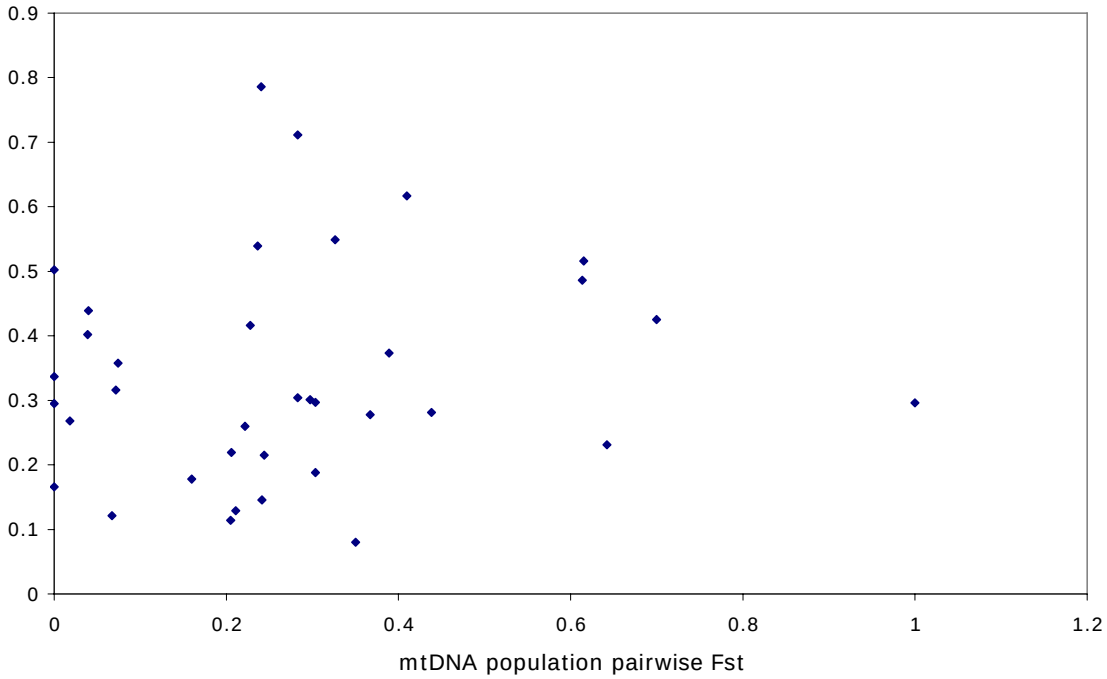



**Figure 6.2** Scatter plot of the microsatellite DNA population pairwise $F_{st}$ and mtDNA population pairwise $F_{st}$ of dugong individuals in the widespread mtDNA lineage.

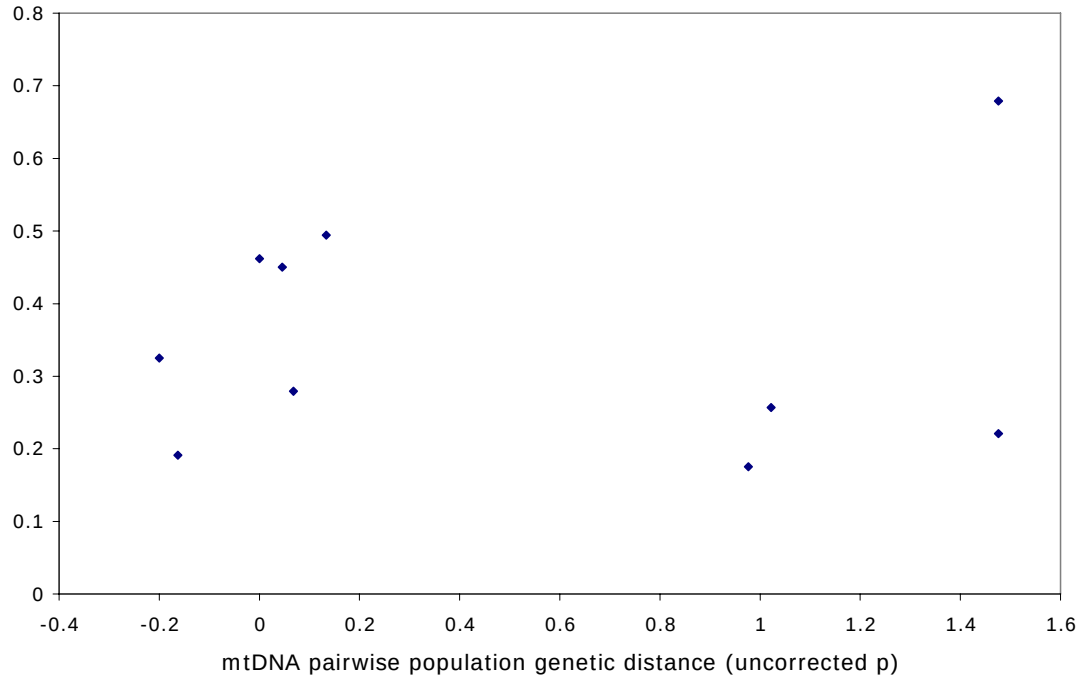



**Figure 6.3** Scatter plot between the microsatellite DNA population pairwise genetic distance and mtDNA population pairwise genetic distance of dugong individuals in the restricted mtDNA lineage.

Section 4.3.5 and section 5.3.4 provide the complete results on the AMOVAs conducted on mtDNA and nDNA data respectively. Definitions of the groupings are outlined in section 2.1.1. A similar lack of concordance is presented when we look at the full datasets for both markers. A summary of the AMOVA results is presented in Table 6.3. Although all AMOVAs conducted in this study were significant (suggesting the dugong population is not panmictic), the nDNA $\phi$-statistics are consistently smaller than the mtDNA $\phi$-statistics. For example, the $\phi_{st}$ for mtDNA when all populations are treated as one region is 0.48, but the equivalent statistic $\phi_{st}$ is lower for nDNA at 0.11. The greatest difference between the mtDNA ($\phi_{sr}$ = 0.37) and nDNA ($\phi_{sr}$ = 0.05) AMOVAs is among populations within regions (Test 2, Table 6.3).

**Table 6.3** Summary of AMOVA results from mtDNA and nDNA based on the full datasets taken from chapters 4 and 5 (mtDNA n = 115; nDNA n = 345).

| Test | Hierarchical Grouping | mtDNA | nDNA |
|---|---|---|---|
| 1 | All populations as one region | $\phi_{st}$ = 0.48 | $\phi_{st}$ = 0.11 |
| 2 | All populations as four regions (Western Australia, North Australia, East Australia, Overseas) | $\phi_{rt}$ = 0.20 (Among regions)<br>$\phi_{sr}$ = 0.37 (Among populations within regions)<br>$\phi_{st}$ = 0.50 (Among populations) | $\phi_{rt}$ = 0.07 (Among regions)<br>$\phi_{sr}$ = 0.05 (Among populations within regions)<br>$\phi_{st}$ = 0.12 (Among populations) |

## 6.4 Discussion

### *6.4.1 Sex-biased dispersal apparent in dugongs*

All analyses conducted on the combined mtDNA and microsatellite results indicate no concordance between the patterns identified in each dataset singly. The strong structure shown from the mtDNA (which is inherited maternally) contrasts the lack of structure from microsatellite DNA (which is inherited from both parents), indicating sex-biased dispersal and/or gene flow. This result disagrees with satellite tracking evidence which shows that both sexes can move long distances (Sheppard *et al.*, in press). However, Sheppard's study is based on a relatively small sample size and dugongs were tracked for periods ranging from 15 days to 551 days, which is a small proportion of their

lifespan. The conflicting results of the genetic and satellite tracking data can be explained: male dugongs are more likely than female dugongs to mate while undertaking these movements. Female dugongs may mate while travelling but return to their 'home' (*i.e.* areas of known seagrass resources) to give birth. This theory supports the philopatry of female dugongs suggested in Chapter 4. If females have knowledge of areas that provide the resources that are required to give birth, they may return to these areas to give birth and while nursing young. It is important to note that male dugongs may also return to known seagrass resources but this cannot be determined from the current data available.

The pattern of male-biased dispersal is common for mammals (Greenwood, 1980; Pusey, 1987; white-tailed deer: Purdue *et al.*, 2000; grizzly bear: Proctor *et al.*, 2004; rufous bettong: Pope *et al.*, 2005; greater long-tailed hamster: Song *et al.*, 2005). Male-biased gene flow has been identified in a number of marine mammals through genetic studies (humpback whales: Baker *et al.*, 1994; Palumbi and Baker, 1994; Baker *et al.*, 1998b; harbour seal: Burg *et al.*, 1999; sperm whale: Lyrholm *et al.*, 1999; Dall's porpoise: Escorza-Trevino and Dizon, 2000; dusky dolphin: Cassens *et al.*, 2005) and a number of fish species (white shark: Pardini *et al.*, 2001; shortfin mako shark: Schrey and Heist, 2003; Patagonian toothfish: Shaw *et al.*, 2004). Differences in movement patterns were identified from satellite-tagging studies in the West Indian manatee with male manatees moving more than female manatees during the six month period that corresponds to the breeding season (Deutsch *et al.*, 2003). During this time male manatees made frequent erratic excursions away from their core area, most probably in search of mates (Deutsch *et al.*, 2003). Interestingly a number of studies incorporating mtDNA and nDNA data for coastal dolphins and porpoises have shown no sex-biased dispersal (Natoli *et al.*, 2005; Rosa *et al.*, 2005). The explanations given for this lack of gender-biased dispersal in dolphins and porpoises is strong social structure, adaptations to local habitat and the lack of resource-driven long distance movements. All of these result in philopatry of both sexes (Natoli *et al.*, 2005; Rosa *et al.*, 2005). In the dugong, non-territoriality, an apparent lack of any strong social bonds, and long distance movements (which are thought to be resource driven) have resulted in a highly complex overlap of individual home ranges (which are estimated to vary from 0.5 $km^2$ to 733 $km^2$ (Sheppard *et al.*, in press)) in which males disperse further than females.

The pattern of lower genetic differentiation identified from nDNA in comparison with mtDNA can also be attributed in part to the haploid, maternal inheritance of mtDNA. This mode of inheritance lowers the effective population size estimated using this marker four-fold relative to diploid nuclear DNA (Birky *et al.*, 1983). However, other factors must be contributing when population differentiation differs by an order of magnitude as it does here (Table 6.3). Another explanation to account for the differences of the mtDNA and nDNA is the high rate of mutation associated with microsatellite loci. Nonetheless the contrasting patterns of $\phi$-statistics that have been presented here strongly suggest male-biased dispersal, which homogenizes allele frequencies among populations at nuclear loci but not mitochondrial markers. This method of interpreting differing patterns between nDNA and mtDNA based on *F*-statistics has been used for humpback whales (Baker *et al.*, 1998b), sperm whales (Lyrholm *et al.*, 1999) and Dall's porpoise (Escorza-Trevino and Dizon, 2000).

### *6.4.2 Interbreeding lineages in dugongs*

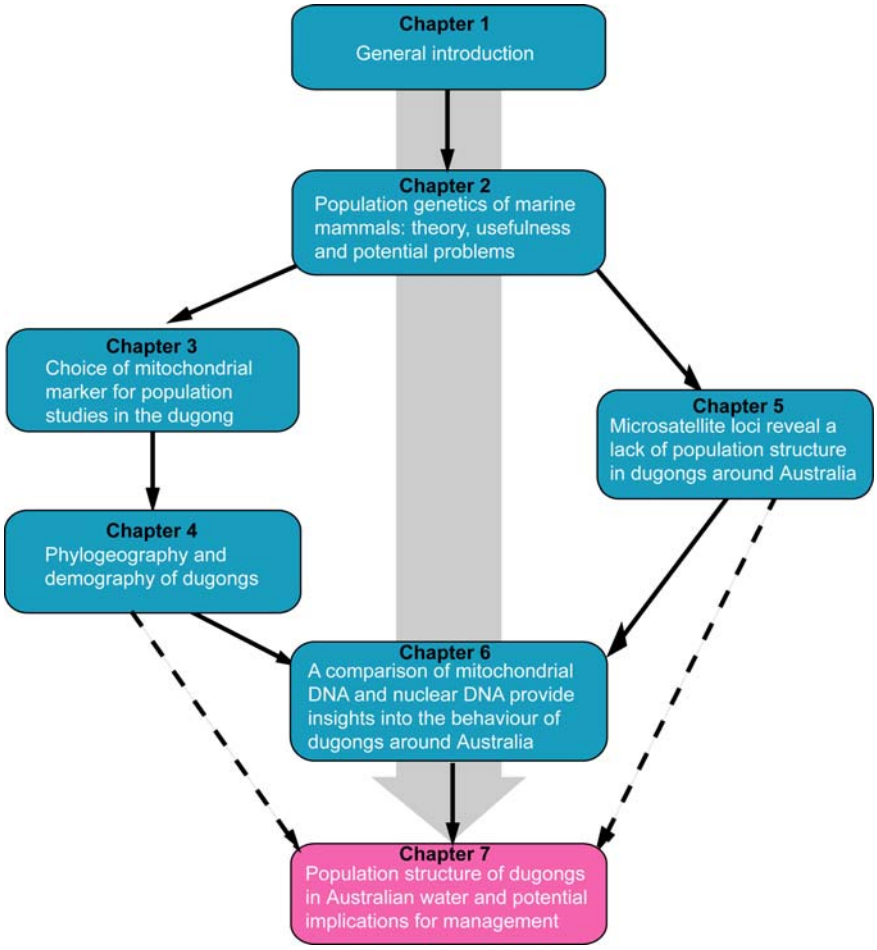
The lack of concordance between the two data sets means the representatives of the each mtDNA lineage identified in Australia are interbreeding with representatives of the other where sympatric. This result suggests that the two refugia at times of low sea level (identified in Chapter 4) were not separated for sufficient time to cause a recognisable difference in appearance or behaviour of the two matrilines, thus facilitating interbreeding between the lineages when reunited. Moritz (1994a) listed two requirements to recognise an evolutionary significant unit (ESU; see section 2.5.1.1): 1) reciprocal monophyly of mtDNA sequences; and 2) significant divergence at nuclear loci. Although the two dugong lineages are distinct at the mtDNA level, they lack divergence at the nDNA loci. Consequently the dugong cannot be split into ESUs around Australia, but could be assigned to geographically based management units (MUs; see section 2.5.1.1). The usefulness of designating management units has been questioned, with many researchers arguing that research priorities should not be based solely on genetic data but should include ecological data as well (Waples, 1998). For dugongs, the management of these MUs is difficult due to the complex pattern of population structure. This complex structure, combined with dugongs' movement abilities, means the scale of management that is required will span political boundaries.

### *6.4.3 Conclusions*

The genetic information obtained in this study clearly indicates that there is a historic signature of population fragmentation due to barriers that were produced at low sea level as indicated by mtDNA. Since these barriers have been removed there has been a high level of gene flow and migration between neighbouring localities with little contemporary population structure as suggested by the microsatellite data. The information obtained from combining the two datasets has shown that female dugongs display some level of philopatry over a broad scale, and male dugongs appear to disperse further from these areas. This male-biased dispersal is common in mammals. The lack of concordance between the datasets also indicates that the two lineages observed in Australia are interbreeding in the areas of overlap. This lack of recent fine-scale population structure and the dugongs' vagility make effective management a challenge. The implications of these results and suggestions as to how they could be implemented for management of the dugong in Australia are looked at in detail in the next chapter.

# Chapter 7: Population structure of dugongs in Australian waters and the management implication

*This chapter summarises the findings of this study as they relate to the management of dugongs in Australia. These findings include the 'healthy' level of genetic diversity present in dugongs, male-biased gene flow, and a high level of gene flow occurring around Australia and connecting Australian dugongs with those in other countries. This high level of gene flow makes it difficult to recognise the allocation of individual management units. These results are compared with other marine species with different ecologies and management protocols. The current legislative framework of dugong management in Australia is summarised and a management regime based on the findings of this work is presented. Future directions for research on this species are also addressed.*



## 7.1 Conclusions drawn from this study as they relate to management

The main reason that the Great Barrier Reef Marine Park Authority supported this study was that they hoped it would provide insights for dugong management. In order to critique and suggest improvements to the current management framework of dugongs in Australia, the results of this study need to be summarised in relation to management. There are four important findings of this study as far as management is concerned: 1) that dugongs appear to be genetically 'healthy'; 2) that there is a significant level of gene flow occurring around Australia, which supports the ecological data available; 3) that no population structure is apparent around Australia making identification of management units (MUs) and evolutionary significant units (ESUs) difficult (see section 2.5.1.1 for definitions); 4) there appears to be gene flow connecting the dugong population in Australia to those in neighbouring countries.

### *7.1.1 Genetically healthy population*

The high degree of genetic variability identified within the dugong for both mtDNA (Chapters 3 and 4) and nDNA (Chapter 5), indicate a 'genetically healthy population'. This conclusion is strengthened when dugongs are compared with their closest extant relative, the Florida manatee. The Florida manatee has fewer haplotypes (Florida manatee - 24 haplotypes in 86 individuals (Garcia-Rodriguez *et al.*, 1998)) compared with the dugong populations I examined (52 haplotypes in 115 individuals, Chapter 4). The Florida manatee also showed lower allelic diversity for nDNA microsatellite loci (Garcia-Rodriguez, 2000; Garcia-Rodriguez *et al.*, 2000) compared with the dugongs I examined (see Table 5.2). Tests on the genetic data indicate that dugongs have not undergone a recent or severe bottleneck (Chapter 5) as evidenced by the high number of alleles observed. The decrease in population numbers of dugongs that have been identified in the last few decades (Marsh *et al.*, 2002, 2005) has not yet left a genetic signature perhaps due to the overlapping generations. In comparison, a small cetacean species that is critically endangered, the vaquita (*Phocoena sinus*), exhibited no variation in the 322 base pairs of the mtDNA control region sequenced in 43 individuals (Rosel and Rojas-Bracho, 1999). This lack of variation was considered to be either a historical effect as a consequence of continuous small effective population size, a severe population bottleneck or founder effect at the time of species' origin. The high level of genetic variation identified within the Australian dugong will allow the population as a

whole to adapt to environmental changes more effectively than species where there is little genetic variation, such as the vaquita.

### *7.1.2 Gender-biased gene flow and dispersal*

Contemporary gene flow in the dugong around Australia is high, with no discrete geographic structuring identifiable in the nDNA (Chapter 5). This result contrasts with the two distinct lineages that were identified using mtDNA (Chapter 4). The biological interpretations of these contrasting results indicate that dugongs that move are mating while travelling. The incomplete overlap in distribution of the mtDNA lineages suggest that although female dugongs may mate while travelling, they potentially return to a home area (which could be large geographically) to give birth. This pattern maintains the incomplete overlap of the mtDNA lineages while homogenizing the nDNA gene flow. As the mtDNA only indicates the female-mediated gene flow within a population, the contrasting results between mtDNA and nDNA may be a result of gender-biased dispersal and gene flow. The higher level of population structuring identified in the mtDNA compared with the nDNA suggest that gene flow is male-biased in dugongs. This phenomenon although common in mammals (Greenwood, 1980; Pusey, 1987), has not been identified in dugongs from satellite tracking studies, with both males and females appearing to undertake long distance movements (Sheppard *et al.*, in press). However, movement differences were identified by satellite tracking of the West Indian manatee by Deutsch *et al.* (Deutsch *et al.*, 2003), with the mature males moving more than mature females. These differences were only identified in the six month period (April to September) which corresponds to the breeding season (Deutsch *et al.*, 2003). It was hypothesised that these frequent erratic excursions away from the core area by male manatees were mate searching expeditions (Deutsch *et al.*, 2003). It is possible that a similar scenario is occurring in the dugongs with male dugongs moving further or more frequently than female dugongs in order to find a mate, but current data are insufficient to support this conclusion.

### *7.1.3 High gene flow makes designation of MUs and ESUs around Australia difficult*

The designation of evolutionary significant units (ESUs) (Moritz, 1994a) is difficult for the dugong as discussed in section 6.4.2. However, two management units could be created as the requirement for this is differentiation at one locus only (Moritz, 1994a). These units would be Western Australia, with only one mitochondrial lineage, and then the rest of Australia, which contains representatives of both mtDNA lineages (Chapter 4). However, the high level of contemporary gene flow among neighbouring localities of dugongs (Chapter 5) would make these management units indistinct entities. This high level of gene flow combined with the high vagility of dugongs, and their variable home ranges, increases the difficulty of assigning discrete management units. These factors and the scale of dugong management required (spanning political boundaries) indicate that co-ordinated management across jurisdictions is needed at local, state and national scales. In addition there is a need to co-ordinate management at an international scale (section 7.1.4).

### *7.1.4 Gene flow connecting Australia and other countries*

Although only a small number of samples were obtained from other countries, the preliminary results of this study indicate there is a significant level of gene flow between Australia and neighbouring countries (section 5.3.4). This result is supported by the presence of a typically Asian haplotype in Ashmore Reef dugongs (Chapter 4) and the lack of strong differentiation at microsatellite loci between Australian and overseas dugongs (Chapter 5). Biologically, the ability of dugong to travel across deep water has been identified at Aldabra Atoll where dugongs, thought to have been absent for 25 years, were recently spotted (Marsh *et al.*, 2002), and by a dugong being sighted at Cocos (Keeling) Islands for the first time (J. Hobbs pers. com. 2005). The movement of only one individual each generation between areas is required to produce genetic homogeneity (Lowe *et al.*, 2004). Given the high vagility of dugongs and the connectivity of countries in Asia, the movement of one dugong each generation [approximately 27 years (H. Marsh pers. com. 2005)] between countries is feasible. This factor emphasises the need for co-ordinated management across areas and political boundaries.

## 7.2 Comparisons with other marine species

Dugongs are different from other marine vertebrates in terms of their ecology and these differences are reflected in their population genetics. Consequently the management of the dugong as a vulnerable species should be appropriate for their ecology and genetics. I give three examples of the genetic structure and management of other marine wildlife and contrast these with the dugong.

### *7.2.1 Case study of loggerhead turtles*

Marine turtles have three distinct life history phases. The first is an oceanic juvenile stage after hatching, older juveniles (subadults) then return to coastal waters, and once mature adult turtles make cyclic migrations from feeding areas to their natal nesting grounds (Bowen *et al.*, 2005). These life stages result in a geographic overlap of demographically independent populations as they mingle at feeding areas and during migration phases (Bowen *et al.*, 2005). These factors result in a complex population structure (Bowen, 1997). By looking at 391 bp in the mtDNA control region of loggerhead turtles (*Caretta caretta*) a step-wise increase in population structure through the life stages is identifiable (Bowen *et al.*, 2005). The oceanic post-hatchling juveniles show no genetic population structure (Bowen *et al.*, 2005). In the subadult loggerhead turtles there is low but significant population structure as turtles recruit into neritic feeding habitats in the vicinity of their natal beaches (Bowen *et al.*, 2005). In contrast, there is strong population structure in the adults as females have high site fidelity to their natal breeding/nesting areas (Bowen *et al.*, 2005). Bowen *et al.* (2005) identified three important lessons for future studies on migratory species:

1) Genetic surveys of migratory species on feeding grounds or migratory corridors may be misleading;
2) Different management regimes for loggerhead turtles are appropriate at different life history stages as different threats are present;
3) Ecosystem-based protection is not sufficient to manage migratory marine species. Rather there should be taxon-specific protection of vulnerable life stages that may differ for different species.

Two important differences are apparent between dugongs and loggerhead turtles: 1) although dugongs are migratory species, their life cycle is much simpler with only three life stages (attendant calf, pre-reproductive juvenile, and adult), all of which live in the same habitats, and 2) although dugongs do move significantly there are no known migratory corridors or movement seasonality; movements are individualistic (Sheppard *et al.*, in press). Thus the management of life history stages as suggested by Bowen is inappropriate for dugongs as no nursery grounds, juvenile feeding habitat or calving grounds have been identified. Therefore a co-ordinated ecosystem approach of protecting valuable resources and habitats for dugongs is more appropriate (see section 7.5.4).

### *7.2.2 Case study of humpback whales*

Humpback whales (*Megaptera novaeangliae*) have historically been divided into stocks: groups of whales using geographically distinct winter ranges for reproduction and non-overlapping summer ranges for feeding (Baker and Palumbi, 1997). However in order to manage the species effectively, studies have been conducted to identify the global population structure using both mtDNA and nDNA (Baker *et al.*, 1993; Baker *et al.*, 1994; Palumbi and Baker, 1994). Humpback whales are highly mobile, making annual migrations (that average 10 000 km per round trip) between summer feeding grounds in high-latitude waters and winter breeding grounds in low-latitude waters, within ocean basins (Baker and Palumbi, 1997). The seasonal opposition of this cycle limits interchange between the Northern and Southern Hemispheres except in areas of overlap near the equator. Thus the three major oceanic populations (North Pacific, North Atlantic and Southern Ocean) are apparently isolated from each other (Baker and Palumbi, 1997). This isolation has been confirmed with mtDNA RFLP and control region sequences (Baker *et al.*, 1993; Baker *et al.*, 1994). Simulations suggested that the minimum number of inter-oceanic migration events necessary to explain the current structure of humpback whales is six in total (Baker and Palumbi, 1997). Further structure is identified within each ocean basin, with populations at distinct feeding grounds travelling to the same wintering areas (Baker and Palumbi, 1997). For example, whales in the Southern Ocean feeding area designated group IV migrate along the coast of Western Australia to wintering grounds on the north west coast of Australia (Baker and Palumbi, 1997). In contrast, whales in feeding group V migrate along the east coast

of Australia or the coastline of New Zealand to different wintering areas (Baker and Palumbi, 1997). A genetic distinction is present between feeding groups IV and V but not between the differing migration corridors within group V (Baker and Palumbi, 1997). This population structuring is unexpected given the mobility and lack of barriers to movement for this species. It has been suggested that the migratory patterns of mothers are transferred to their young (Baker and Palumbi, 1997). In Australia, management initiatives for humpback whales focus on the protection of migration corridors along either coast by the production and reinforcement of guidelines to mitigate the human-induced effects on migrating individuals. The important difference between the humpback whales and dugongs with respect to management is the predictability of the humpback's migratory habits. The apparent temporal and spatial unpredictability of dugong movements (Sheppard *et al.*, in press) make management of migratory corridors or distinct feeding/breeding areas inappropriate. However, there are feeding areas which consistently support large numbers of animals. Protecting these areas can be an effective management strategy as discussed in Grech and Marsh (in review).

### *7.2.3 Case study of coastal dolphins*

Two coastal dolphins found in Australia, Australian snubfin dolphin (*Orcaella heinsohni*) and the Indo-Pacific hump-backed dolphin (*Sousa chinensis*), have a similar distribution to the dugong but very different ecology. Although very little information is available for the coastal dolphins, their similarities to dugongs in habitat distribution make worthwhile comparisons. In Australia, the Australian snubfin dolphin and Indo-Pacific humpback dolphins have been recorded around Northern Australia, including the coastal waters of Queensland, Northern Territory, and Western Australia (Parra, 2005). Throughout their range the Australian snubfin dolphin occurs not more than 10 km from land, in water less than 10m deep and within 10 km from river mouths (Parra *et al.*, 2002). The Indo-Pacific humpback dolphin also occurs in the shallow coastal waters within 10 km from the coast (Great Barrier Reef Marine Park Authority, 2000; Parra, 2005). Neither of these coastal dolphin species are considered migratory (Jefferson and Leatherwood, 1997; Great Barrier Reef Marine Park Authority, 2000). However, both species have been thought to undergo limited seasonal movements (Jefferson and Leatherwood, 1997; Great Barrier Reef Marine Park Authority, 2000).

Population behavioural studies indicate that there is strong site fidelity and limited movement between bays (Parra, 2005). This result is supported by preliminary genetic data suggesting discrete population units (Hale *et al.*, 1998; Hale *et al.*, 2004). When these units are better understood, they will presumably form the basis for MUs for these species. This result contrasts significantly with the data from dugongs presented in this study, where MUs cannot be identified, although the habitats of all three species are very similar.

These case studies demonstrate that the management of marine wildlife needs to be appropriate for each species' behaviour, ecology, and genetics. Appropriate dugong management requires the development of co-ordinated large-scale management plans that can then be adjusted at the smaller scale for local threats and community needs. Community participation is particularly important in areas where traditional hunting of dugongs is a Native-Title right. For this large-scale co-ordinated management to be successful the patchwork of laws and current management plans that occur within Queensland and around Australia needs to be refined.

## 7.3 Current legislative framework for dugong management in Australia

In order to develop management regimes for dugongs in Australia the framework of legislation that applies to the dugong needs to be understood at several jurisdictional levels (Figure 7.1). The international commitments that Australia has made to protect the dugong are the first level, followed by federally administered legislation in Australia. As the dugong is distributed across the coastal waters of two states and one territory in Australia, each state then has a number of statutes that address the conservation of the dugong. Scaling down another level, marine parks have legislation associated with them and a number of local Indigenous communities have put in place dugong management plans. This hierarchical arrangement of laws and legislation is illustrated in Figure 7.2.

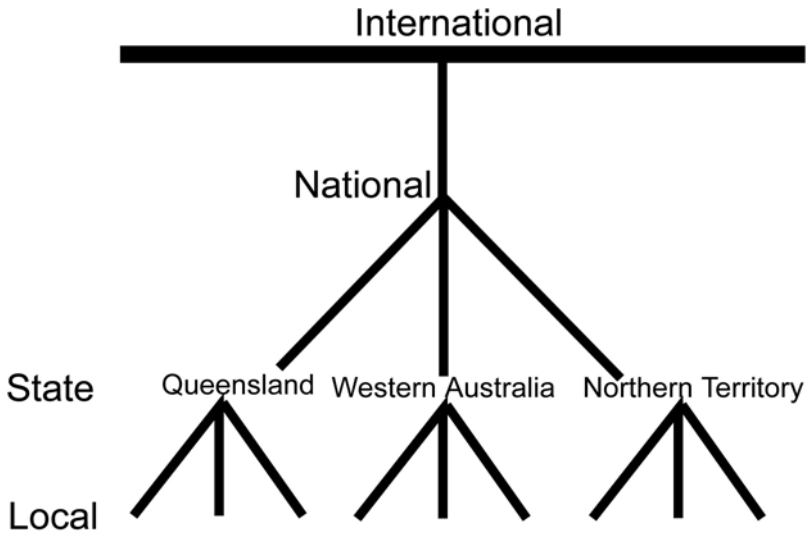



**Figure 7.1** Schematic diagram indicating the various jurisdictional levels of the law relating to dugong management within Australia. Refer to Figure 7.2 for the relevant legislation at each level.

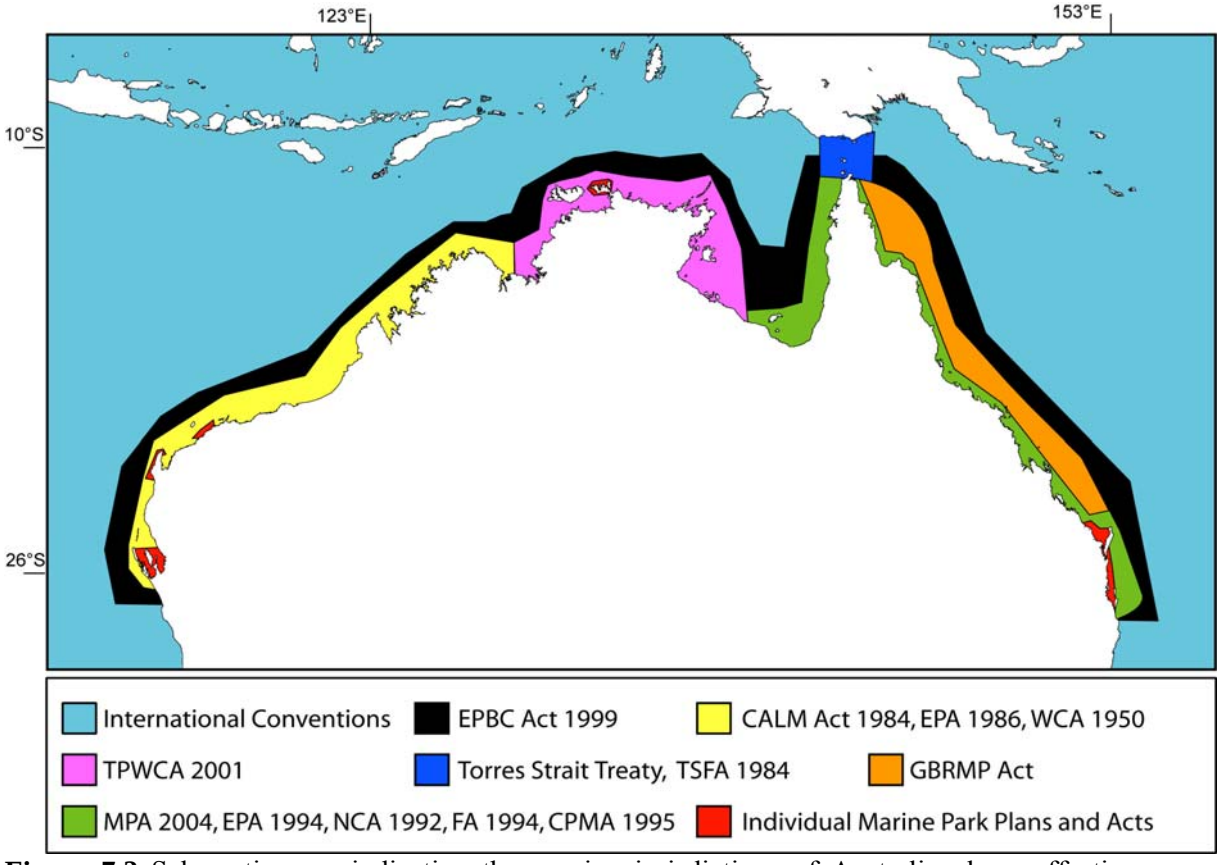



**Figure 7.2** Schematic map indicating the varying jurisdictions of Australian laws affecting dugong management. Jurisdictions not to scale and overlaps not shown. Actual situation is more complex than presented here. 'MPA' = Marine Parks Act, 'EPA' = Environmental Protection Act, 'NCA' = Nature Conservation Act, 'FA' = Fisheries Act, 'CPMA' = Coastal protection and management Act, 'GBRMP' = Great Barrier Reef Marine Park, 'TSFA' = Torres Strait Fisheries Act, 'TPWCA' = Territory Parks and Wildlife Conservation Act, 'CALM' = Conservation and Land Management, 'WCA' = Wildlife Conservation Act, 'EPBC' = Environmental Protection and Biodiversity Conservation.

### *7.3.1 International conventions relating to dugong conservation and management*

Australia is the only developed nation in the dugong's range to have a substantial dugong population. The other developed country in their range is Japan with a very small dugong population (Marsh *et al.*, 2002). It is therefore important for Australia to play a major role in the global conservation of the dugong. Australia is a signatory to several international conventions on the conservation of the environment including: the *Paris Convention Concerning the Protection of the World Natural and Cultural Heritage (1972)*, the *Declaration of the United Nations Conference on Environment and Development (1992),* and the *United Nations Convention on Biological Diversity (1992)*. The dugong is listed under Appendix II (which suggests it is a species that needs or would significantly benefit from international co-operation) of the *Convention of Migratory Species* to which Australia is a party. Similarly, the dugong is listed under Appendix I of the *Convention on International Trade in Endangered Species* (CITES), to which Australia has been a signatory since 1976. CITES is an agreement between nations that aims to ensure that international trade in flora and fauna does not threaten their survival.

### *7.3.2 Federally administered laws that relate to dugongs*

Dugongs are listed as both a marine species under section 348 and as a migratory species under the *Environment Protection and Biodiversity Conservation Act (1999)* (*EPBC Act*). It is an offence to kill, injure, take, trade, keep, or move any dugong on Commonwealth land or in Commonwealth waters without a permit. It is also illegal to take action that has, will have or is likely to have, a significant impact on a listed migratory species without approval from the Environment minister. An additional objective of the *EPBC Act* is "*to provide for the protection of the environment, especially those aspects of the environment that are matters of national environmental significance*". Matters of national significance include the Commonwealth marine environment, World Heritage Areas, nationally threatened species, and migratory species protected under international agreements (Stokes and Dobbs, 2001). One of the reasons the Great Barrier Reef was listed as a World Heritage Area is the large population of dugongs that it supports (Lucas *et al.*, 1997). Similarly, Shark Bay in Western Australia, where a substantial dugong population resides, is also listed as a

World Heritage Area. The dugong is therefore a species of national significance as it is listed under international agreements as addressed previously (section 7.3.1) and is of world heritage value. The provisions in the *EPBC Act* provide a good basis for overall protection of dugongs in Australia.

The *Great Barrier Reef Marine Park Act (1975)* and it's regulations which apply to the 340 000 $km^2$ of the Great Barrier Reef Marine Park, which extends along the Queensland coast, offshore from the tip of Cape York peninsular to just north of Bundaberg, are federal statutes the object of which is to make provision for the establishment, control, care and development of a marine park in the GBR area. This act established the Great Barrier Reef Marine Park Authority. Protection is provided to dugongs in the marine park through zoning, issuing of permits and implementation of plans of management that collectively enable management of human activities (Stokes and Dobbs, 2001). Threatened species are further managed for their 'recovery and continued protection and conservation' (Stokes and Dobbs, 2001). This protection of resources may provide a suitable network of protection to mobile marine mammals such as the dugong (see section 7.5.4).

### *7.3.3 Current legislative framework at the state level*

Dugongs are distributed around Northern Australia across two states and on territory, *i.e.* along the coast of Queensland, the Northern Territory and Western Australia. Therefore I will address the legislative framework for each state separately.

#### *7.3.3.1 Queensland*

Relevant legislation for the state of Queensland includes the *Nature Conservation Act 1992 (Qld)*, *The Nature Conservation (Wildlife) Regulations 1994 (Qld)*, E*nvironmental Protection Act 1994 (Qld)*, *Coastal Protection and Management Act 1995 (Qld)*, *The Fisheries Act 1994 (Qld)*, and the *Marine Parks Act 2004 (Qld)*. The objective of the *Nature Conservation Act 1992 (Qld)* is the conservation of nature to be achieved through seven methods outline in the act. The relevant procedures to dugongs are the '*protection of native wildlife and its habitat and the protection of the biological*

*diversity of native wildlife and its habitat'* and the '*use of protected wildlife and areas to be ecologically sustainable'*. The *Nature Conservation (Wildlife) Regulation 1994 (Qld)* lists the dugong as a vulnerable species requiring the government to put into effect recovery plans or conservation plans for wildlife and their habitat (Stokes and Dobbs, 2001). Other responsibilities of the government concerning vulnerable species include establishing databases and records about the species, educating the community, preserving viable populations in the wild and regularly monitoring and reviewing the species' conservation status (Stokes and Dobbs, 2001).

Within Queensland, Torres Strait is governed by a number of additional laws. The *Torres Strait Treaty* between Papua New Guinea and Australia defines the boundaries between the two countries and how the sea area may be used. This Treaty establishes the Torres Strait Protected Zone to allow the Torres Strait Islanders and the coastal people of Papua New Guinea to carry on their traditional way of life. The establishment of the Torres Strait Protected Zone has helped to preserve and protect the land, sea, and air of the Torres Strait including the animal life (www.dfat.gov.au/geo/torres_strait). However, the dugong is a subject of an unregulated and unsustainable traditional fishery in this area (Heinsohn *et al.*, 2004; Marsh *et al.*, 2004). The *Torres Strait Fisheries Act 1984 (Qld)* aims to protect the resources of both traditional and commercial fisheries. Part 5 Article 20 of this Act states that '*A Party may adopt a conservation measure consistent with the provisions of this Part which, if necessary for the conservation of a species, may be applied to traditional fishing, provided that that Party shall use its best endeavours to minimise any restrictive effects of that measure on traditional fishing*'. The wording of the Torres Strait Fisheries Act is weak, with words such as 'may' and 'if necessary'. Traditional fishing is given precedence over the persistence of the target species. Based on current scientific data (Heinsohn *et al.*, 2004; Marsh *et al.*, 2004) a strategic assessment of the risks of traditional hunting is currently occurring under the *EPBC Act*. It is vital to understand the connection between the conservation of culturally important species and the traditional way of life. If the species is not conserved than the way of life is also lost.

#### *7.3.3.2 Northern Territory*

The dugong is listed as protected wildlife in the Northern Territory under section 43 of the *Territory Parks and Wildlife Conservation Act 2001* (TPWCA). It is prohibited to take, interfere with, possess, control or move protected wildlife, unless authorised to do so under the Act. Authorisation can be obtained by way of a permit issued by Director of the Parks and Wildlife Commission of the Northern Territory. The TPWCA recognises the rights of Aboriginal peoples who have traditionally used an area of land or water to continue to use that area for traditional hunting, non-commercial food gathering and for ceremonial and religious purposes. Traditional hunting of dugongs is allowed under the Act. The TPWCA also allows parks and reserves to be declared and the management of dugong populations to be addressed through specific park plans of management. In the Northern Territory only one marine park has been declared, the Cobourg Peninsula Aboriginal Land, Sanctuary and Marine Park. This marine park has its own legislation (*Cobourg Peninsula Aboriginal Land, Sanctuary and Marine Park Act 2000*) requiring that a plan of management be prepared regarding the protection, conservation and management of native fauna, which would include dugongs. A "draft management program for the dugong (*Dugong dugon*) in the Northern Territory of Australia" was written in 2003, however I have been unable to confirm the status of this plan.

#### *7.3.3.3 Western Australia*

In Western Australia, three acts relate to dugong conservation and management. They are the *Conservation and Land Management Act 1984 (WA)*, *Environmental Protection Act 1986 (WA)*, and the *Wildlife Conservation Act 1950 (WA)*. Under the *Wildlife Conservation Act* the environment minister lists protected species (*Wildlife Conservation (Specially Protected Fauna) Notice 2005*). Dugongs are listed under schedule 4. The CALM Act has designated marine parks however only three marine parks are in the dugong's range. They are the Shark Bay Marine Park, the Ningaloo Marine Park, and the Rowley Shoals Marine Park.

## 7.4 Ideal management framework

This study indicates that there is gene flow occurring between Australia and other countries. It is therefore important for Australia to take a significant role in the co-ordinated conservation of dugongs internationally. A strong federal stance on management and conservation of dugongs will clearly state Australia's position on the conservation of dugongs worldwide. Additionally co-operation especially between neighbouring countries such as Papua New Guinea is vital. The current international conventions and agreements including the Torres Strait Treaty provide an appropriate framework in which to develop initiatives.

The legislative framework that affects dugongs in Australia is complex (section 7.3), and needs refining in order to better manage this vulnerable species. The most important factor is co-operation between the different jurisdictional levels to ensure a consistent management scheme over the entire population. This study has shown that dugongs around Australia compose one interconnected stock. Thus co-operation is required between the Commonwealth, Queensland, Northern Territory and Western Australia. Federally the dugong is listed as a migratory and marine species to be conserved, however it is listed as vulnerable in Queensland, is the subject of an unregulated and unsustainable fishery in Torres Strait and is listed as protected in the Northern Territory and Western Australia. These differences in the conservation status of a population which is essentially one stock is confusing. One priority for dugong conservation in Australia should be keeping a connected population that is not fragmented more than that which naturally occurs. For instance, if the dugongs residing in the Gulf of Carpentaria were continually extirpated, than the two groups on either side of this gap would eventually diverge genetically. If management and conservation status were consistent across the range of dugongs in Australia than the likelihood of such a fragmentation occurring would be lower. The federal government has both the power and the precedent to take a leading role in dugong management bit has not done so to date, although current initiatives are promising (see section 7.5).

The second area that requires further development is the enforcement of legislation. Effective enforcement is challenging for the dugong, which occurs in very remote areas

in Australia, making the resources required to effectively enforce the legislation extremely expensive. The Native-Title right to hunt further complicates the enforcement. All the legislation that conserves the dugong explicitly does not extinguish the Native-Title rights of traditional owners including those to hunt dugongs. There is great difficulty in proving that Indigenous people have stepped outside the bounds of their actual or presumed Native-Title rights. In order to overcome this problem, local scale management plans that limit the take of dugongs could be entered into with traditional owners. The difficulty with such plans will be enforcement and co-ordination across jurisdictions. However, if the community elders are behind such a management scheme they may help the enforcement of such programs by traditional means.

## 7.5 Current initiatives to improve management

There are currently several initiatives being undertaken by the Australian government to improve the management of dugongs. These include the international "regional dugong conservation memorandum of understanding", the national framework for Indigenous harvest, North Australian Indigenous Land and Sea Management Alliance dugong and marine turtle project, the GBRMP representative areas program (RAP) and traditional use marine resource agreements (TUMRAs).

### *7.5.1 International Convention on Migratory Species*

A draft of the international 'regional dugong conservation memorandum of understanding (MoU)' was written after the first meeting on dugong conservation in the Indian Ocean and Southeast Asian region under the sponsorship of the Convention on Migratory Species (CMS) in August 2005. Regional frameworks such as these provide an opportunity for countries to cooperate to conserve species, to share information, and to seek financial and technical resources between countries with similar goals. MoUs are non-legally binding frameworks that reflect the aspirations of the Signatory States. However, as these aspirations do not necessarily translate to effective on-ground actions in some instances, this meeting emphasised a strong focus on ensuring that MoUs deliver on-ground conservation actions for the dugong. I have shown that there is gene flow between Australia and neighbouring countries (section 5.3.4), therefore this

regional initiative is an important step in advancing the international co-operation to conserve dugongs.

### *7.5.2 National framework for Indigenous harvest*

In 2005 Australia produced the *Sustainable harvest of marine turtles and dugongs in Australia – A national partnership approach.* This document indicates that some harvests are sustainable while others may be unsustainable. The migratory nature of dugongs, and the lack of genetic structure revealed in this study, mean that the unsustainable harvest in one area is likely to impact other areas. A list of a range of actions to address the unsustainable and illegal harvest of marine turtles and dugongs is outlined in the draft plan. However no budget or timelines have are included in this plan. This national partnership approach of sustainable harvest is an important start to co-ordinating the management and conservation of dugongs in Australia, but it is not the solution to all the problems. Particularly the challenge of co-ordinating management across biologically (national), logistically (state) and culturally (local) relevant scales. There also needs to be a co-ordinated program of research and management to improve the information and knowledge of dugongs. As such, there are a number of questions that my study has identified which should be addressed on a national scale (section 7.6).

### *7.5.3 North Australian Indigenous land and sea management alliance*

The North Australian Indigenous Land and Sea Management Alliance dugong and marine turtle project was developed in 2005, to co-ordinate the regional activity involving dugong and marine turtle around Northern Australia. The alliance consists of five representative groups (the Kimberley Land Council, the Northern Land Council, the Carpentaria Land Council Aboriginal Corporation, the Balkanu Cape York Development Corporation and the Torres Strait Regional Authority) that represent the Indigenous communities of Northern Australia. This alliance has short-term funding for the activities proposed. In order to be ensure continual success, further funding and a longer term approach should established. However, this alliance is an excellent opportunity for Indigenous communities and governments (local, state and federal) to co-ordinate management of dugongs and marine turtles for future generations.

### *7.5.4 Great Barrier Reef Marine Park representative areas program (RAP) and traditional use marine resource agreements (TUMRAs)*

*Great Barrier Reef Marine Park Zoning Plan 2003* lists dugongs as a protected species. This zoning plan implements the representative areas program (RAP), where the aim was to maintain biological diversity by optimising the design of a network of no-take areas that covered the range of habitats and communities found within the GBRWHA (Day *et al.*, 2000). Recent studies on the usefulness of the RAP and the GBRMP zoning plan suggest that 85% of dugongs in the GBRWHA occur in areas with a high level of protection from identified anthropogenic impacts (Grech and Marsh, in review). This level of protection for the dugong in the GBRWHA is adequate and the representative areas program (although not specifically designed to protect dugongs) could be a model used by other states and marine parks to conserve dugongs.

The *Great Barrier Reef Marine Park Zoning Plan 2003* also establishes a method of managing the traditional use of marine resources through the development and implementation of Traditional Use of Marine Resources Agreements (TUMRAs). TUMRAs are voluntary agreements developed by Traditional Owners and aim to promote indigenous stewardship and co-management of the habitats of protected species (Havemann *et al.*, 2005). TUMRAs are area based rather than species based. Although they provide a good start to the local management of marine resources the migratory habits and connectivity of dugong populations (as shown in this study) mean that the TUMRA scheme may not provide the appropriate scale of dugong management for effective conservation. Effective conservation might only be achieved by the incorporation of species specific local and regional management plans along side the TUMRA scheme. This study identified that there is high connectivity of neighbouring dugong populations; therefore it is vital that there is co-ordination across adjacent TUMRA areas.

The Great Barrier Reef Marine Park Authority has two policies for dugongs, one for the urban coast or Southern GBR, and one for the Northern GBR. Although I have shown that dugong populations are genetically connected along the coast of Queensland, I believe that the two policies for dugongs in the Great Barrier Reef are justified because

of the different threats dugongs encounter in these two areas. Along the urban coast (the Southern GBR) the main threats are destruction of habitat, boat strikes, and run off from farming practices. Whereas, in the Northern GBR the Indigenous hunting will have the most impact on the dugong population. These different threats do require different management regimes as has been implemented by the Great Barrier Reef marine Park Authority.

## 7.6 Conclusions and future research

This study has used both mtDNA and nDNA to gain information on the population genetic structure of dugongs around Australia. Identification of appropriate sections of the mtDNA genome in dugongs for phylogeographic and demographic analyses identified that most of the variation was contained within the control region (Chapter 3). This region was found to be phylogenetically useful by analyses of the dugong's closest relatives the Paenungulata (Chapter 3). These analyses consistently grouped species into the known orders of the paenungulates (Hyracoidean, Proboscidea and Sirenia) although the relationships between these orders were unclear (Chapter 3). Future research is required to understand fully the relationships between these orders, including the development of an appropriate molecular clock to identify the time of divergence between these very morphologically distinct but genetically similar orders. These studies should include additional loci (possible suggestions include nuclear intron sequences) and representatives from all genera and all orders.

The 5' end of the mtDNA control region was then used in the phylogeographic study of dugongs. This study indicated that historic habitat fragmentation (caused by the presence of land bridges at times of low sea level) resulted in the differentiation of two distinct lineages in Australia (Chapter 4). These lineages expanded in distribution once the Torres Strait land bridge submerged, and currently overlap (Chapter 4). Future management plans should focus on maintaining a continuous habitat for the dugong to avoid further differentiation of dugong lineages. Because of the low sample numbers obtained from outside Australia the number of distinct lineages occurring outside Australia and the relationship of these lineages to those in Australia is unknown. Future research should look at including additional samples from Australia's neighbouring

countries to enhance the knowledge of the causes and relationships between the mtDNA lineages in the dugong, and establish historical isolations and connectivity.

Analyses of nDNA identified significant levels of gene flow between geographic locations (Chapter 5). However, all six microsatellite loci used in this study failed to comply with Hardy-Weinberg expectations across all demes (Chapter 5). The most probable cause for this is non-random mating within the dugong (Chapter 5). Future research should focus on the interrelatedness of feeding herds and mating systems of dugongs to identify if non-random mating is responsible for the non-compliance to the Hardy-Weinberg equilibrium.

Comparison of nDNA and the mtDNA lineages indicate that the two distinct lineages are interbreeding in the areas they overlap in distribution (Chapter 6). The information obtained from the two genetic data sets also indicates that gene flow within dugongs is male-biased (Chapter 6). As the available ecological data on dugong movements indicates that both sexes undertake long distance movements further research into this behaviour is required.

This study has provided valuable information into the biology and ecology of dugongs around Australia. Important findings for management are:

1) Dugongs appear genetically healthy with a high level of variability found in the mtDNA and nDNA,
2) There is a high level of gene flow occurring around Australia with no identifiable genetically distinct populations that can be designated into management units,
3) Gene flow is male-biased,
4) There is connectivity between the dugong population of Australia and neighbouring countries.

Although additional research is required to answer the questions raised by this study, the co-ordination of management procedures across large geographical scales (state, national and international scale) should be progressed immediately. This study has shown that within Australia the dugongs compose one large interconnected stock and that management protocols will need to be co-ordinated at international, national, regional and local scales if they are to be effective.

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# Appendices

**Appendix 1** A summary table of a number of genetic studies on marine mammals indicating the species studied, marker used and if population differentiation was identified.

| Paper | Organism | Marker used | Population Differentiation Identified |
|---|---|---|---|
| Allen *et al.* (1995) | Grey Seal (*Halichoerus grypus*) | Microsatellites | Yes, between breeding sites in Britain |
| Andersen *et al.* (1998) | Atlantic Walrus (*Odobenus rosmarus rosmarus*) | Microsatellites & mtDNA RFLPs | Yes, between East Greenland, West Greenland and Franz Joseph Land-Svalbard |
| Andersen *et al.* (2003) | Minke Whale (*Balaenoptera acutorstrata*) | MtDNA control region sequences & microsatellites | Yes, between West Greenland, Central North Atlantic. North East Atlantic and the North Sea |
| Baker *et al.* (1998a) | Humpback whale (*Megaptera novaeangliae*) | mtDNA control region | Yes, within the Southern Hemisphere |
| Baker *et al.* (1999) | Southern Right Whale (*Eubalaena australis*) | mtDNA control region | Yes, between wintering grounds |
| Bakke *et al.* (1996) | Minke Whales (*Balaenoptera acutorostra*) | mtDNA control region | Yes, within the Antarctic and between the Nth Atlantic and Antarctic but not within the North Atlantic |
| Bickham *et al.* (1996) | Stellar Sea Lion (*Eumetopias jubatus*) | mtDNA control region | Yes, between the west coast of America and Russia |
| Boskovic *et al.* (1996) | Grey Seals (*Halichoerus grypus*) | RFLP | Yes, within Atlantic, North West Europe and the Baltic |
| Burg *et al.* (1999) | Harbour Seal (*Phoca vitulina*) | mtDNA control region & microsatellites | Yes, within British Columbia |
| Dalebout *et al.* (2001) | Northern Bottlenose Whales (*Hyperoodon ampullatus*) | MtDNA control region sequences | Yes, in the Western North Atlantic |
| Dowling and Brown (1993) | Bottlenose Dolphin (*Tursiops truncatus*) | RFLP | Yes, between Atlantic and Pacific populations |
| Fullard *et al.* (2000) | Long-finned pilot whales (*Globicephala melas*) | Microsatellites | Yes, within the North Atlantic. Between West Greenland and remaining sites |
| Garcia-Martinez *et al.* (1995) | Striped Dolphin (*Stenella coeruleoalba*) | RFLP | Not around the Spanish Mediterranean coast |
| Garcia-Martinez *et al.* (1999) | Striped dolphin (*Stenella coeruleoalba*) | RFLP | Yes, between the Mediterranean and Atlantic |
| Gladden *et al.* (1999) | Beluga whale (*Delphinapterus leucas*) | Microsatellites | Yes, the Western Arctic was differentiated from other locations studied |

**Appendix 1** continued over page.

**Appendix 1** continued.

| | | | |
|---|---|---|---|
| Goerlitz *et al.* (2003) | Eastern North Pacific Gray Whales (*Eschrichtius robustus*) | mtDNA control region sequences | Yes, female philopatry to natal lagoons was detected |
| Goodman (1998) | European Harbour Seals (*Phoca vitulina vitulina*) | Microsatellites | Yes, within Europe |
| Goodwin *et al.* (1996) | Bottlenose Dolphin (*Tursiops truncatus*) | Allozymes | Yes, from cluster analysis differences in the North and South population are present. However no significant differences in $F_{st}$ values. |
| Harlin *et al.* (2003) | Dusky Dolphin (*Lagenorhynchus obscurus*) | mtDNA control region sequences | No differentiation among the four regions around New Zealand |
| Hoelzel *et al.* (1998) | Bottlenose Dolphin (*Tursiops truncatus*) | Microsatellites, mtDNA & simple sequence copy polymorphism | Yes, within the Western North Atlantic |
| Kappe *et al.* (1995) | Harbour seal (*Phoca vitulina*) | Random Amplified Polymorphic DNA & minisatellites | Not between the Dutch Wadden sea and Scotland |
| Kretzmann *et al.* (2001) | Hawaiian Monk Seal (*Monachus schauinslandi*) | Microsatellites | Yes, between the extreme ranges of the Hawaiian islands |
| Lazaro *et al.* (2004) | Franciscana Dolphin (*Pontoporia blainvillei*) | mtDNA control region sequences | Yes, along the southeast coast of South America. |
| Lento *et al.* (1994) | New Zealand Fur Seals (*Arctocephalus forsteri*) | Cytochrome b gene | Yes, between West Australian and New Zealand Rookeries |
| Lyrholm and Gyllensten (1998) | Sperm whale (*Physeter macrocephalus*) | mtDNA control region | Yes, between ocean regions but not within the Atlantic |
| Maldonado *et al.* (1995) | California Sea Lions (*Zalophus californianus*) | mtDNA control region & Cytochrome b gene | Yes, between the Pacific coast and Gulf of California |
| Mizuno *et al.* (2003) | Spotted Seal (*Phoca largha*) | mtDNA sequences - control region & tRNA | No geographic structure along the coast of Hokkaido, Japan. |
| Natoli *et al.* (2004) | Bottlenose dolphin (*Tursiops* spp.) | mtDNA control region & microsatellites | Yes at a global/regional scale with both mtDNA and microsatellites |
| Palsboll *et al.* (1995) | Humpback whale (*Megaptera novaeangliae*) | mtDNA control region | Yes, within the Atlantic |
| Palumbi and Baker (1994) | Humpback whale (*Megaptera novaeangliae*) | Nuclear introns | No obvious geographic structure between California and Hawaii |
| Pichler *et al.*(1998) | Hector's Dolphin (*Cephalorhynchus hectori*) | mtDNA control region sequences | Yes, around New Zealand |
| Rosel and Rojas-Bracho (1999) | Vaquita (*Phocoena sinus*) | mtDNA control region | No, sequences showed very little differentiation |

**Appendix 1** continued over page.

**Appendix 1** continued.

| | | | |
|---|---|---|---|
| Schaeff *et al.* (1997) | North and South Atlantic Right Whales (*Eubalaena glacialis* and *australis*) | Minisatellites | Yes, between the species |
| Secchi *et al.* (1998) | Franciscana (*Pontoporia blainvillei*) | mtDNA control region sequences | Yes, along the coast of Brazil |
| Slade *et al.* (1998) | Southern Elephant Seal (*Mirounga leonina*) | mtDNA control region, nuclear DNA sequences and microsatellites | Yes, within the Antarctic |
| Stanley *et al.* (1996) | Harbour Seals (*Phoca vitulina*) | mtDNA control region | Yes, on a regional scale, between and within oceans |
| van Vauuren *et al.* (2002) | Heaviside's dolphin (*Cephalorhynchus heavisidii*) | mtDNA control region sequences | Not between South Africa and Namibia |
| Walton (1997) | Harbour Porpoise (*Phocoena phocoena*) | mtDNA control region | Yes, around the united Kingdom |
| Wang *et al.* (1996) | Harbour Porpoise (*Phocoena phocoena*) | RFLP | Yes within the North Atlantic |
| Whitehead *et al.* (1998) | Sperm Whale (*Physeter macrocephalus*) | mtDNA control region | No |
| Yoshida and Kato (1999) | Bryde's Whale (*Balaenoptera edeni*) | mtDNA control region & Cytochrome b gene | Yes, between oceans |

**Appendix 2** Complete alignment of the dugong control region. B61 - dugong from Townsville, MD56 and T677 (Sequence from Tikel 1997) from Torres Strait, SW1 - Dugong from Ashmore Reef, D3 - Dugong from Moreton Bay (sequence from Tikel 1997), LEM1 - Dugong from the Philippines, GB - Sequence obtained from GenBank (Accession Number NC_003314), GB2 - Sequence obtained from GenBank (Accession Number AY075112). Conserved Sequence Blocks, Extended Termination Associated Sequence, L-strand promoter and origin of H-strand replication regions are underlined. The three domains of the control region are identified with the central conserved domain sequence in bold. A '.' indicates similarity with the top sequence (B61), '-' indicates a gap and '?' indicates missing data.

```
5′ domain >
     1                                                                                                ETAS1   100
B61  GCGCGCGCTATGTACTTCGTGCATTACGTGCTCCTCCCCATAATAGTACTATATATGTTTTATCTTACATACACCATCCTATGTATAATCGTGCATTACA
MD56 ........................T......T.........C.....................................................................
T677 ........................T.....................................................C.....................
SW1  ........................T......T.........C.............C.........................................
D3   ........................T......T.........C.....................................................................
LEM1 ........................T......T.........C.....................................................................
GB   --......................T.....................................................................................
GB2   .......................T......T.........C.....................................................................

    101      ETAS1                                                                                                200
B61  CTACTTACCCCATGCATATAAGCCAGTACAGTAGGATTCATGCTCTAAAGCCTAAGTAATTAATCTCCATTATACAACCTCTACACCATGGATATTGTCC
MD56 ..................................................CC..C.....C...........C.................
T677 ..........................G..........................................................................
SW1  ..............G.......T.................................A.G..C..C.....C...........CG.................
D3   ..................................................A...CC..C.....C...........C.................
LEM1 ..............G.......T.....................C.........ACG.CC.....T..C...........C.................
GB   ............................G..........................................................................
GB2  ..................................................A...CC..CT....C...........C.................

    201                                                ETAS2                                                300
B61  AGTCCATGTACCTCTTGATCTTGCATAGTACATTCAACCCTTTGTCGTACATAGCACATCTCTGAGATAGTTCTCGTCAACACGCTTATCACCTCCAATG
MD56 ....................................A.............................C..T.....................
T677 ..........T.....................................A.....................................................
SW1  ..................................T.....................................................................
D3   ......................................T.....A.............................C..T.....................
LEM1 ..C.............................T.....................................................................
GB   ..........T.....................................A.....................................................
GB2  ....................................A.............................C..T.....................
```

**Appendix 2** Complete alignment of dugong control regions continued.

```
   301                                        <5′ domain CC domain >                                    400
B61  AACAGTCCTTGACTACCAAGCTTCGAGAAACCAGCAACCCGCTCCGATTATGCTTCTCTTCTCGCTCCGGGCCCATAACTTGTGGGGGTGTCTACACTGA
MD56 G.....................................................C.....................G.....................
T677 .....................................................C.....................................................
SW1  G.....................................................C.....................G.T..................
D3   G.....................................................C.....................G.T..................
LEM1 G.....................................................................T.....................
GB   .....................................................C.....................................................
GB2  G.....................................................C.....................G.T..................

   401                                                                                                 500
B61  ATCTATACCTGGCATCTGGTTCTTTCTTCAGGACCATCTCACCTAAATTCGCCCACTCTTTCCCCTTAAATAAGACATCTCGATGGACTTATGACTAATC
MD56 ................................G.....................................................................
T677 ................................G.....................................................................
SW1  ..................K.............G.....................................................................
D3   ................................G.....................................................................
LEM1 ................................G.....................................................................
GB   ................................G.....................................................................
GB2  ................................G.....................................................................

   501                                                <CC domain 3′ domain >                            600
B61  AGCCCATGATCATAACATAACTGTGATGTCATGCATTTGGTATCTTTTAATTTTCGGGATGCAACGACTCAACTAGGCCGTCTGAGGCCTTAACACAGGC
MD56 .....................................................................................................
T677 ....................................................................................................-....
SW1  .....................................................................................................
D3   ....................................................................................................-....
LEM1 .....................................................................................................
GB   .....................................................................................................
GB2  .....................................................................................................
```

**Appendix 2** Complete alignment of dugong control regions continued.

```
     601                                                                                                  CSB1                 700
B61  AAATAACTTGTAGCTGAACCCAGATTGAATATTAAGTACTGGCGCCATTTACCATAAGGTGTTATTCAGTCAATGCTCGAGGACATAGAATTTACCAAAC
MD56 ..................................................................................................................
T677 ....................................C.............................................................................
SW1  ..................................................................................................................
D3   ..................................................................................................................
LEM1 ..................................................................................................................
GB   ..................................................................................................................
GB2  ..................................................................................................................

   701                                                                                                                1146
B61  CCCATTTCCCAG(CGCATA)63-------------------------------------------------------------------------------------------
MD56 ............(CGCATA)2(CA)3(CGCATA)5(CA)3(CGCATA)60.....................................................................
T677 .........-..(CGCATA)41.....................................................................................................
SW1  ............(CGCATA)67.....................................................................................................
D3   .........-..(CGCATA)41.....................................................................................................
LEM1 ............(CGCATA)3CACATACACGCA(CGCATA)4CACATACACGCA(CGCATA)7CACGCA(CGCATA)6CACGCA(CGCATA)5CACGCA(
GB1  ............(CGCATA)10CACGCA(CGCATA)7CACGCA(CGCATA)7CACGCA(CGCATA)17CACGCA(CGCATA)19.................
GB2  ............(CGCATA)2(CA)3(CGCATA)4(CA)3(CGCATA)2CTGCATACGCATACACGCA(CGCATA)5CACGCA(CGCATA)41(GGCATA

   1147                                                  CSB2                                                        1247
B61  -----------TATACGTATGTTAAACCACAGAATTATCTCTCACAAACCCCCCTACCCCCCTTAATTACCCTTAACTAGGTTTCTATAAGTATTTTTTT
MD56 ..................................................................................................................
T677 ..................................................................................................................
SW1  ..................................................................................................................
D3   ..................................................................................................................
LEM1 CGCATA)40.........................................................................................................
GB   ..................................................................................................................
GB2  )3(CGCATA)2.....................................................T..........................................
```

**Appendix 2** Complete alignment of dugong control regions continued.

```
      1248   CSB3                                                                LSP                        1347
B61   AATCTTGTCAAACCCCAAAAGCAAGATATACTACAGAAAGTAAGGGTACGGGTAAACTATACAGACCAGCCCGCTAACACTTAACCAATTGAGATAATTC
MD56  ..........................................................................................................
T677  ..........................................................CG..............................................
SW1   ..........................................................................................................
D3    ..........................................................CG..............................................
LEM1  ..........................................................................................................
GB    ..........................................................................................................
GB2   ..........................................................................................................

      1348                                                                                                  1447
B61   CTTTTTT-CCCGCTAATACCGATATACCACTTAAATAGTTTTATTTCCTTTTTAAGAGCTATGTTCCTAGATTTGAAACTCACAGGTCTTACAATTTGGA
MD56  ..........................................................................................................
T677  ..........................................................................................................
SW1   ..........................................................................................................
D3    ..........................................................................................................
LEM1  ..........................................................................................................
GB    ..........................................................................................................
GB2   .......T..................................................................................................

       1448         <3′ domain 1471
B61   TCGAAACGGGG-CCCCGCTGGTTCAT
MD56  ..........A..............
TG77  ..........A..............
SW1   ...........G.............
D3    ..........A..............
LEM1  .........................
GB    .........................
GB2   ..........A..............
```

**Appendix 3** Paenungulate alignment two. '.' Indicates similarity to top sequence. '?' indicates missing data. '-' Indicates an alignment gap or deletion. B61, MD56, LEM1, SW1, D3, T677, GB1 and GB2 are dugong control region sequences. T. man = Florida manatee; T. man2 = Florida manatee (from GenBank); T. inu = Amazonian manatee; L. afr = African elephant; E. max = Asian elephant; D. dor = Tree hyrax; P. cap1 = Rock hyrax (from GenBank); P. cap2 = Rock hyrax sequenced in this study.

```
B61      CGCGCGCTATGTACTTCGTGCATTACGTGCTCCTCCCCATAATAGTACTATATATGTTTTATCTTACATACACCAT-CCTATGTATAATCGTGCATTACACTACTTACCCCATGCATATA
MD56     ........................T......T.........C.....................................................................................
LEM1     ........................T......T.........C.....................................................................................
SW1      ........................T......T.........C............C.....................................................................G....
D3       ........................T......T.........C.....................................................................................
T677     ........................T.....................................................C...............................................
GB1      ........................T......T.........C.....................................................................................
GB2      ........................T......T.........C.....................................................................................
T. man   -TACG........A...........T...T..T........-.........C.......A.................................A.....................T....
T. man2  -TACG........A...........T...T..T........-.........C.....................................A.........TA.........T....
T. inu   -TACG........A.............................-.........C.......A.....G.....................A.........TA.........T....
L. afr   AA.C.........TA..........AA....TG........C..T.....AC...AC..A..........G.....A.-..................C...T..T...........T....
E. max   AATC..........A..........AA.....G........C..A.....AC...AC..A..........G.....A..-..................C...T..T...........T....
P. cap   ------------------ACA.GA....A.C..C......C-------.C.CGC....A.AT..AC.GC---------.........-.........-.ACTG...................
P. cap2  ????????????????????????????????????????????????????????????????????????????????????????????????????????????????????
D. dor   --TC.TACCAAC.AAAAAC....A----CTC..CG....C.----------------------.GG-GC---------.........-.........-.A.T..TC.G.............

B61      AGCCAGTACAGTAGGATTCA-TGCTCTAAAGCC-TAAGTAATTAATCTCCATTATACAACCTCTACACCATGGATATTGTCCAGTCCATGTACCTCTTGATCTTGCATAGTACATTCAAC
MD56     ....................................A...CC..CT....C...........C.....................................................
LEM1     ...T.....................C..........ACG.CC.....T..C...........C.....................C.............................T...
SW1      ...T.....................................A.G..C..C.....C...........CG.....................................................T...
D3       ....................................A...CC..CT....C...........C.....................................................-
T677     .........G.....................................................................................T.....................
GB1      ....................................A...CC..CT....C...........C.....................................................
GB2      ....................................A...CC..CT....C...........C.....................................................
T. man   ...A.........T..G.-.A.....CT.GA.AG..TA..-CC---T....C.GC.G.TT.C.A..CA..........C.T........TC.TTC......A........C....CATG.
T. man2  ...A.........TA-GA..A.....CT.GA.AG..TA..-CC---T....C.GC.A.CT.C.A..CA..........C..........T...TC...A..A........C...C.ATGT
T. inu   ...AG....G...CA.G.-.A.....CC.GA.AA..TA..-CC---T....C.G..A.TT.C.A..CA..........C.T........T...TC......A........C....CAT..
L. afr   ...A.....T..TTA.-CT.A..TGTC..GT.A...TTC.TGT.GA-.T..CAGGT..TGT...GGTT..........A.TT.CCTACGA..AAC.A.AG....A.....C.....A..G
E. max   ...A.....T..TTA-G...A..TGTC..GT.A...TT.GTGT.GA-....CAGGTT.TGT....GTT.............T..CCTACGA..AAC.A.AG....A.....C.....A..G
P. cap   ...A......-..-A.G...A..TA.A...A.CC...A--CCAT.AAC.A-CA...A....CAA...A....AC..A--------------------------CA.G.AC.A.A.A...
P. cap2  ????????????????????????????????????????????????????????????????????????????????????????????????????????????????????
D. dor   ...T......-..-A.G...A..TATATC.A.TAC..AA--CC..-AC...CAT..A.-..A.A.T.A..C.AC..A--------------------------C..C.AC.A.A...GA
```

**Appendix 3** Paenungulate alignment two continued.

```
B61     -CCTTTGTCGTACATAGCACATCTCT-GAGATAG-TTCTCGTCAACACGCTTA-TCACCTCCAATGAACA-GTCCTTGACTACCAAGCTTCGAGAAACCAGCAACCCGCTC-CGATTATG
MD56    ......A.............................C..T.............................G.....................................................C.
LEM1    ..................................................................G.....................................................
SW1     ..................................................................G.....................................................C.
D3      T.....A.............................C..T.............................G.....................................................C.
T677    ......A.....................................................................................................................C.
GB1     ......A.............................C..T.............................G.....................................................C.
GB2     ......A.............................C..T.............................G.....................................................C.
T. man  T....AA..............TA..T..A..CA..C..........T..............T.AGG...........TC.......GC.....................C..TC..CT..
T. man2 T....AA..............A..T..A..CA.....T.C.....T..............T.AGG...........G........GC.....................C..TC...T..
T. inu  CT...AA....C...........A..T..A..CA.............T..............T.AGG...........TC.......GC.....................C..TC...T..
L. afr  CT...G..............A.......A.T.C...A....C..T..A..............GTTG.TA....A......T.C..C..........T........C.ATCT.CG..
E. max  CT...GA...........G...TA.......A.T.....A....T..T..A............C.GTTG.TAT...A......T.C..C..........T........C.ATCT.CG..
P. cap  .--.GAA..AAG..C..G...CAA.....C.A.AA...AA.C.......GA..A..T.TA......GGA.ATTTA..A.TC.....CTCA..T....T...........C.ATC.A-G.A
P. cap2 ??????????????????????????????????????????????????????????????????????????????????????????????????.........C.A.T.AA-.A
D. dor  .--.GAA.-.ACT...AG...CAA.....CTA.AAC..CA.A........A............CCAGGAG.TCTA..A......T.C..C..T..............C.A.TGAA-..

B61     CTTCTCTTCTCGCTCCGGGCCCATAACTTGTGGGGGTGTCTACACTGAATCTATACCTGGCATCTGGTTCTTTCTTCAGGACCATCTCACCTAAATTCGCCCACTCTTTCCCCTT-AAAT
MD56    ........................G.....................................................G.....................................
LEM1    .........................T.....................................................G.....................................
SW1     ........................G.T.....................................................K.....G.....................................
D3      ........................G.T.....................................................G.....................................
T677    ..........................................................................G.....................................
GB1     ........................G.....................................................G.....................................
GB2     ........................G.....................................................G.....................................
T. man  TCC.....................................A.....................................................................................
T. man2 TCC.....................................??????????????????????????????????????????????????????????????????????????????
T. inu  TCC.....................................??????????????????????????????????????????????????????????????????????????????
L. afr  TCC.....................C.A.........T....T...G.....................................................A.......T.......T........
E. max  TCC.....................C.A.........T....T...G.....................A.....................A.....................T........
P. cap  TCC.......................AC........AG...A...........T..A...........A.....................A..........A.............
P. cap2 TCC.......................A.........AG...G.............................A.....................A..........A....T...T....
D. dor  ..CTCT....................A.........AG...AC...........T..A.........C.A........??????????????????????????????????????
```

**Appendix 3** Paenungulate alignment two continued.

```
B61     AAGACATCTCGATGGACTTATGACTAATCAGCCCATGATCATAACATAACTGTGATGTCATGCATTTGGTATCTTTT-AATTTTCGGGATGCAACGACTCAACTA-GGCCGTCTGAGGCC
MD56    ....................................................................................................................
LEM1    ....................................................................................................................
SW1     ....................................................................................................................
D3      ....................................................................................................................-
T677    ....................................................................................................................-
GB1     ....................................................................................................................
GB2     ....................................................................................................................
T. man  ..................................................G.........................G.......C.....................
L. afr  ..............T.A.....................................G.....................T.....G.......TGT..T...G...T.............
E. max  ..............T.A.....................................G.....................T.....G.......TGT..T...G...T.............
P. cap  ................A.................C.....................................T.-....G.......TGT..TC....................
P. cap2 ................A.................C.................G.................T....T.-....G.......TGT..TC....................
D. dor  ????????????????????????????????????????????????????????????????????????????????????????????????????????????

B61     TTAA-CACAGGCAAATAACTTGTAGCTGAACCCAGATTGAATATTAAGTACTGGCGCCAT-TTACCAT--AAGGTGTTATTCAGTCAATGCTCGA-GGACATAGAATTT-ACCAAACCCC
MD56    ....................................................................................................................
LEM1    ....................................................................................................................
SW1     ....................................................................................................................
D3      ....................................................................................................................
T677    ..............................................C.....................................................................
GB1     ....................................................................................................................
GB2     ....................................................................................................................
T. man  C.........T....C..............TA.....C..............CC..CAT.C.............................G..A........T......-
L. afr  C.........T...GC............G.TTGA.....G.......AT.....A.GG.A-..-T....GG...A..................GA.......A.....T-TT.....T-
E. max  C.........T...GC............G.TTGA.....G.......AT.....A.GG.A-..-T....GG...AC.................GA............T-TT.....T-
P. cap  .GGGGTT...T.................G...TT.........T.--C...C.AG...TA....TAATGTT..................T..A.......A-T.CACGTTG.-T.T-
P. cap2 .GGGGTT...T.................G...TT.........T.--C...CTAG...AA....TAATGTT.........................----------------.T-
D. dor  ????????????????????????????????????????????????????????????????????G..-.......G..K........NG-AAT.CAC.TT..-..T-
```

**Appendix 3** Paenungulate alignment two continued.

```
B61      A--TTTCCCTATACGTATGTTAAA-CCAC---AGAATTATCTCTCACAAACCCCCC--TACCCCCC--TTAA--TTACCCTTAACTAGGTTTCTATAAGTAT-TTTTTTAATCTTGTCAA
MD56     ....................................................................................................................
LEM1     ....................................................................................................................
SW1      ....................................................................................................................
D3       ........-...........................................................................................................
T677     ........-...........................................................................................................
GB1      ....................................................................................................................
GB2      ..........................................................T.........................................................
T. man   .AA..-...--CG.A..C.....-....T...G.........A..........A........A.................G....A.............--......T.........
L. afr   .AA..-...........C..-.....-.....TATGT..C.......T-..........T.............G....T....GG..CAA........G.G..A......T...C......
E. max   .AA..-...........C..-.....-.....TATGT..C.......T-..........T.............G.........GG..CAA........G....A......T...C......
P. cap   .AA..-.....-A-C.ACA..-CA....ACT..G-..TG..TA.G.........CC........CCG..CTC......-A..T..-------..C.....CA..AGC--.C........
P. cap2  .AA..-.-------Y-G.....-.CA...SACT..G-..TGT.TA.G.........CC........CCG..CTC......-A..T..--------------.CA..AGC--.C........
D. dor   .AA..-..-------.-G.....-.CA.G..AST..G-..TGT.TA.G.....A....CC......T.TTGG.CTT......-A..T..--------------.CA..AGC--.CA...G...

B61      ACCCCAAAAGCAAGA-TATACT-ACAGAAAGTAAG--G-G--TACGGGTAAACTATACAGACCAGCCCGCT-AACA-CTT----AACCAATTGAGATAATTCCTTTTTT-CCCGCTA-AT
MD56     ....................................................................................................................
LEM1     ....................................................................................................................
SW1      ....................................................................................................................
D3       ..........................................................CG........................................................
T677     ..........................................................CG........................................................
GB1      ....................................................................................................................
GB2      ....................................................................................................................T.........
T. man   ......................T....--TG.GAAA.-.A......AC......C.A.A.TT----....T.-.TAG.ATTTT..TT.....W..C..C.....-....-......
L. afr   -...........G.-C........T....--TG..ACCAA.AG.....-...................G.GAG..AT...TAG..ACAT.-GGGG.GA.AGC..A..A......-.G..-T.CG..
E. max   ...........G..C........T....--TG..ACCAA.AG..GATA.G...-----------G.GAG..AT...TAG..ACAT.-GGGG.GA.AGC..A.TA......-.G..-T.CG..
P. cap   .............GC.....CT...T...TG..AAA.T-AC...---------....CGAG..GATA-----.----..AT..ATACC..ACC.AA.C.C....---------..A.-......
P. cap2  .............GC......T...T...TG..AAA.T-AC...---------....CGAG..GATA-----.----..AT..ACACCC.ACC.AA.C.C....---------..A.-T..G-.
D. dor   .....??????????????????????????????????????????????????????????????????????????????????????????????????????????????????

                                B61      ACCGATATACCACTTAAATAGTTTTATTTCCTTTTTAAGAGCTATGTTCCTAGATTTGAAA
                                MD56     ............................................................
                                LEM1     ............................................................
                                SW1      ............................................................
                                D3       ............................................................
                                T677     ............................................................
                                GB1      ............................................................
                                GB2      ............................................................
                                T. man   ...AG...G..C...T...TAA....C..T..............CA.C.......C.....
                                L. afr   ..TTG-G...T.......A.AA.......TT....C......C.................
                                E. max   ..TTGGG...T.......A.AG.......T.....C......CT................
                                P. cap   ...CG............GGG....C.C..TACAC.A......C....C....CG.C-....
                                P. cap2  ...C..............G....C.C..TACA..AG....AC....C....CG.C-.T..
                                D. dor   ????????????????????????????????????????????????????????????
```

**Appendix 4** Sample locations grouped into broad regional areas with the respective number of individuals collected for each location listed with their haplotypes my dataset (n = 115; 492 bp). If more than one individual from a location had the same haplotype, the number is indicated in parentheses. The total number of samples from each region is indicated in parentheses. The haplotypes have been placed into their respective lineages.

| Location | Latitude and longitude | Sample number (n) | Haplotypes present | | |
|---|---|---|---|---|---|
| | | | Widespread lineage | Restricted lineage | Other lineages |
| **Western Australia** | | (28) | | | |
| Ashmore Reef | 12.20S, 123.10E | 3 | 44 | | 50 (2) |
| Beagle Bay | 16.56S, 122.32E | 3 | 23 (2), 48 | | |
| Cape Lambert | 20.64S, 117.74E | 1 | 44 | | |
| Exmouth | 22.20S, 114.09E | 5 | 19, 23, 44 (2), 45 | | |
| Shark Bay | 26.00S, 113.30E | 15 | 03, 19 (3), 20 (2), 21, 22, 23 (2), 24, 25, 26, 27, 28 | | |
| Roebuck Bay | 17.58S, 122.15E | 1 | 29 | | |
| **Northern Australia** | | (33) | | | |
| Blue Mud Bay | 13.42S, 136.03E | 4 | 18 (2), 32 | 31 | |
| Darwin | 12.23S, 130.44E | 4 | 06, 45, 46,49 | | |
| Torres Strait | 10.07S, 142.07E | 25 | 03, 06 (3), 08, 10, 12, 13, 14 (2) | 04, 05 (7), 07 (2), 09 (2), 11 (3), | |
| **North Queensland** | | (15) | | | |
| Cardwell | 18.27S, 146.02E | 5 | 02 (2), 18 (2), 32 | | |
| Cooktown | 15.48S, 145.25E | 1 | 18 | | |
| Hopevale | 14.49S, 144.58E | 1 | 18 | | |
| Ingham | 18.65S, 146.16E | 1 | 02 | | |
| Townsville | 19.16S, 146.49E | 7 | 01, 02 (3), 18, | 31 | |
| **South Queensland** | | (30) | | | |
| Hervey Bay | 24.57S, 152.40E | 6 | | 30 (2), 31 (2) 47, 52 | |
| Moreton Bay | 27.25S, 153.20E | 14 | 39 | 30, 31 (6), 35 (2), 36, 37, 38 | 34 |
| Shoalwater Bay | 22.22S, 150.23E | 10 | 18 (4), 32, 39 | 30, 31 (2) 33, | |
| **Asia** | | (9) | | | |
| Indonesia | 03.43S, 128.12E | 1 | | | 51 |
| Japan | 26.33N,127.83E | 3 | | | 41, 42, 43 |
| Thailand | 08.04N,098.52E | 3 | | | 15 (2), 17 |
| Philippines | 10.47N,119.32E | 1 | | | 16 |
| Sabah | 06.88N,116.83E | 1 | | | 40 |

**Appendix 5** Variable base positions identified in the long haplotypes (492 bp; n = 115). '.' Indicates similarity with the first sequence. 'Hap' = Haplotypes. '-' indicates a gap and '?' indicates missing data. Lineages into which haplotypes belong are indicated. 'W' = widespread lineage; 'R' = Restricted lineage; 'O' = Other lineages.

| *Hap.* | *Lineage* | *Base Position* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1111111111 | 1112222222 | 2222222222 | 2222233333 | 334444444 | |
| | | 1133788 | 1145556677 | 7890001112 | 2222333334 | 4456733455 | 563667777 |
| | | 1490258689 | 4751450967 | 8606784692 | 4589123481 | 3695317556 | 828230289 |
| 01 | W | CCAGTAAGTC | GACGGAAGAG | GAGTGGGAAG | GAAATACTGT | AGTGTAAAGG | GGGCGGAT- |
| 02 | W | .......... | .......... | ....A..... | .......... | .......... | ......... |
| 03 | W | .......... | .......... | ....A..... | .......... | ..C....... | ......... |
| 04 | R | ...ACGG.C. | AGT....... | ........G. | AGGG..T... | .......GA. | .A.....-. |
| 05 | R | ....C.G.C. | AGT....... | ........G. | AGGG..T... | .......GA. | .A....... |
| 06 | W | ......G... | ..T......A | ....A..... | .......... | ..C....... | .A....... |
| 07 | R | ...A..G.C. | AGT....... | ........G. | AGGG..TC.. | .......GA. | .A....... |
| 08 | W | ......G... | .......... | ....A..... | .......... | .......... | .A....... |
| 09 | R | ...ACGG.C. | AGT....... | ........G. | AGGG..T... | .......GA. | .A....... |
| 10 | W | .A........ | .......... | .......... | R......... | ..Y....... | .A....... |
| 11 | R | ...A.G..C. | AGT....... | ........G. | AGGG..T... | .......GA. | .A....... |
| 12 | W | ......G... | .......... | ....AA.... | .......... | .......... | .A....... |
| 13 | W | ......G... | .......... | ....A..... | ........A. | .......... | .A....... |
| 14 | W | ......G... | .......... | ....A..... | .......... | ..C....... | .A....... |
| 15 | O | ...AC...CT | ..T.A..A.. | ........GA | .G.G..T... | ....CG.G.. | .A....G.. |
| 16 | O | ...A....C. | ....A..... | .G......GA | ..GGCGT... | G..AC..GA. | .A...???? |
| 17 | O | ...AC.G.C. | ....A..... | ...C....GA | .GG.CGT... | G..AC..GA. | .A....... |
| 18 | W | ......G... | .......... | .......... | .......... | .......... | .A....... |
| 19 | W | .......... | .......... | ....A..... | .......... | ..C....... | .......-. |
| 20 | W | .......... | .......... | A...A..... | .......... | ..C....... | ......... |
| 21 | W | .......... | ..T....... | ....A..G.. | .......... | ..C....... | ......... |
| 22 | W | ..C....... | .......... | ....A..... | .......... | .......... | ......... |
| 23 | W | .......... | .......... | ....A..... | .......... | ..C....... | .A....... |
| 24 | W | .......... | .....G.... | ....A..... | .G........ | .......... | .A.....-. |
| 25 | W | .......... | .......... | A...A..... | .......... | .......... | ......... |
| 26 | W | ..C....... | .......... | A...A..... | .......... | ..C....... | ......... |
| 27 | W | ..T...G... | .......... | ....A..... | .......... | ..C....... | .A......T |
| 28 | W | ....N..... | .....G.... | ....A..... | .G........ | ..C....... | .A....... |
| 29 | W | ......G... | .......... | ....A..... | .......... | .......... | .A......T |
| 30 | R | ...AC.G.C. | AGTA....GA | ........G. | AGGG..T... | .......GA. | .A......T |
| 31 | R | ...AC.G.C. | AGTA...... | ........G. | AGGG..T... | .......GA. | .A....... |
| 32 | W | ......G... | ..T.....GA | ....A..... | .......... | ..C....... | .A....... |
| 33 | R | ...AC.G.C. | AGT...G... | ........G. | AGGG..T... | .......GA. | .A....... |
| 34 | O | T...C.G.C. | ANC....... | .......... | A.N...N... | .......NA. | .A.....?? |
| 35 | R | ...AC.G.C. | AGT....... | ........G. | AGGG..T... | .......GA. | .A....... |
| 36 | W | ......G... | .......... | .......... | .......... | .......... | .A...A.?? |
| 37 | R | ...AC.G.C. | AGTA...... | ........G. | AGGG..T... | .......GA. | .AA.TN-?? |
| 38 | R | ??.AC.G.C. | AGTA...... | ........G. | AGGG..T... | .......GA. | .A.T....T |
| 39 | W | ......G... | ..T......A | ....A..... | .......... | .AC......A | .A....... |
| 40 | O | ...AC.G.C. | ....A..... | ........GA | .GG.C.T... | G..AC..GA. | .A....... |
| 41 | O | ...-..G.C. | ....A..... | .G......GA | ..GGCGT... | G..AC..GA. | .A...-.?? |
| 42 | O | ...A..G.C. | ....A..... | .G......GA | ..GGCGT... | G..AC.TGA. | .A...-..? |
| 43 | O | ......G.C. | ....A..... | ...C....GA | ..GGCGT... | ...AC..GA. | AA.....?? |
| 44 | W | .......... | .......... | ....A..... | .G........ | ..C....... | .A....... |
| 45 | W | .......... | .......... | .......... | .......... | .......... | .A......T |
| 46 | W | ......G... | ..T....... | ....A..... | .......... | .......... | .A...-.?? |
| 47 | W | ......G... | ..T......A | ....A..... | .......... | .AC......A | .A...-.?? |
| 48 | W | .......... | ..T....... | ....A..... | .......... | ..C....... | ......... |
| 49 | W | ......G... | ..T......A | ..A....... | .......... | ..C....... | .A....... |
| 50 | O | ...AC.G.C. | ....A..... | ...C....G. | .GG.C.T... | ...ACG.GA. | .A....... |
| 51 | O | ...AC...C. | ....A..... | ........GA | .GG.CGT..C | G..AC..GA. | .A....... |
| 52 | R | ?????.GTC. | AGTA...... | ........G. | AGGG..T... | .......GA. | .A....... |

**Appendix 6** Table showing all variable sites and haplotypes for the dataset containing Tikel (1997) sequences (n = 212; 192bp). Haplotypes numbers followed by DT are haplotypes identified by Tikel (1997). Haplotype number without DT were identified in this study. Many unique haplotypes dissolved into more common haplotypes when the shorter region was used therefore these haplotypes are not shown below. The lineage that contains the haplotypes are shown. '.' Indicates similarity with the top sequence. Base position numbers are indicated. 'W' = Widespread lineage; 'R' = Restricted lineage; 'O' = Other lineages.

| *Haplotype* | *Lineage* | *Base positions* | | | | |
|---|---|---|---|---|---|---|
| | | 11244477 | 111111 8889000011 | 1111111111 3333444445 | 1111111111 5555566666 | 1111111 6777899 |
| | | 4867512539 | 2387145648 | 4569023470 | 2367901268 | 9014703 |
| DT04 | R | GATCGCAGTG | GAAGAAGGAG | TGGGTGTAGG | AGGGTACTGT | TTAGTAG |
| DT05 | W | ......GA.. | ......A... | .A......A. | GAAA....A. | ....C.. |
| DT07 | W | .......A.. | ......A... | .A......A. | GAAA...... | ....... |
| DT08 | R | ......GAC. | .......... | .A......A. | GAAA...... | ....... |
| DT13 | W | ..C....A.. | ......A... | .A......A. | GAAA...... | ....C.. |
| DT16 | R | ..C......A | .......... | .......G.. | ......T... | ....... |
| DT17 | W | ......GAC. | .......... | .A......A. | G.AA.....C | ....C.. |
| DT18 | R | .........A | .......... | ........G. | ......T... | ....... |
| DT20 | W | ......GA.. | ......A... | ........A. | GAAA...... | ....C.. |
| DT21 | O | ..C...GAC. | A......... | C.......A. | G..AC.T... | ..G...A |
| DT23 | W | ......GA.. | ......A... | .A......A. | GAAA...... | .C..C.. |
| DT24 | R | .GC......A | .....GA... | .......... | ......T... | ....... |
| DT26 | W | .......AC. | .......... | .A........ | GAAA...... | ....... |
| DT28 | O | ..CT.TGA.. | A..A...... | .....A...A | G.A...T... | ....... |
| DT30 | R | ..C...GA.A | .......... | .......... | ......T... | ....... |
| DT31 | W | ....A.GAC. | .......... | ........A. | GAAA...... | ....... |
| DT32 | R | ..C......A | .....GA... | .......... | ......T... | .....G. |
| DT33 | R | ..C......A | .....GA... | .......... | ......T... | .C..... |
| DT35 | O | ..C....AC. | .......... | ...AC...A. | ..A..GT... | ....... |
| DT36 | W | ......GAC. | ....T..... | ........A. | GAAA...... | ....... |
| DT37 | W | ......GA.. | ...A...... | .A......A. | GAAA...... | ....C.. |
| DT38 | O | ..C...GAC. | .......... | .......... | G....GT... | ..G...A |
| 01 | W | ......GAC. | .......... | ........A. | GAAA...... | ....... |
| 02 | W | ......GAC. | .......... | .A......A. | GAAA...... | ....... |
| 03 | W | ......GAC. | .......... | .A......A. | GAAA...... | ....C.. |
| 04 | R | ..C....... | .......... | .......... | ......T... | ....... |
| 06 | W | ......GA.. | ......A... | .A......A. | GAAA...... | ....C.. |
| 07 | R | ..C....... | .......... | .......... | ......TC.. | ....... |
| 10 | R | ..C......A | .......... | ......C... | ......T... | ....... |
| 12 | W | ......GAC. | .......... | .AA.....A. | GAAA...... | ....... |
| 13 | W | ......GAC. | .......... | .A......A. | GAAA....A. | ....... |
| 15 | O | ..CT..GA.. | A..A...... | .....A...A | G.A...T... | ....... |
| 16 | O | ..C...GAC. | A.......G. | .........A | GA..CGT... | ..G...A |
| 17 | O | ..C...GAC. | A......... | C........A | G..ACGT... | ..G...A |
| 20 | W | ......GAC. | .......A.. | .A......A. | GAAA...... | ....C.. |
| 21 | W | ......GA.. | .......... | .A.....GA. | GAAA...... | ....C.. |
| 22 | W | ......GAC. | .......A.. | .A......A. | GAAA...... | ....... |
| 24 | W | ......GAC. | .G........ | .A......A. | G.AA...... | ....... |
| 28 | W | ......GAC. | .G........ | .A......A. | G.AA...... | ....C.. |
| 30 | R | ..C......A | .....GA... | .......... | ......T... | ....... |
| 31 | R | ..C......A | .......... | .......... | ......T... | ....... |
| 32 | W | ......GA.. | .....GA... | .A......A. | GAAA...... | ....C.. |
| 33 | R | ..C....... | ..G....... | .......... | ......T... | ....... |
| 34 | O | ..C....NC. | .......... | ........A. | .ANA..N... | ....... |
| 39 | W | ......GA.. | ......A... | .A......A. | GAAA...... | ...AC.. |
| 40 | O | ..C...GAC. | A......... | .........A | G..A..T... | ..G...A |
| 43 | O | ..C...GAC. | A......... | C........A | GA..CGT... | ......A |
| 44 | W | ......GAC. | .......... | .A......A. | G.AA...... | ....C.. |
| 46 | W | ......GA.. | .......... | .A......A. | GAAA...... | ....... |
| 48 | W | ......GA.. | .......... | .A......A. | GAAA...... | ....C.. |
| 49 | W | ......GA.. | ......A..A | ........A. | GAAA...... | ....C.. |
| 50 | O | ..C...GAC. | A......... | C......... | G..A..T... | ......A |
| 51 | O | ..C...GAC. | A......... | .........A | G..A.GT... | C.G...A |
| 52 | R | T.C......A | .......... | .......... | ......T... | ....... |

**Appendix 7** Frequency of alleles in each population for each locus. '-' indicates alleles not present in population.

| Locus | Allele | Shark Bay | North WA | Northern Territory | Torres Strait | North Qld | Central Qld | South Qld | ASIA | PACIFIC |
|---|---|---|---|---|---|---|---|---|---|---|
| **TmaE08** | **168** | - | - | - | - | 0.03 | - | - | - | - |
| | **170** | 0.13 | 0.04 | - | 0.07 | 0.03 | 0.04 | - | 0.11 | 0.33 |
| | **172** | 0.82 | 0.89 | 0.88 | 0.72 | 0.72 | 0.59 | 0.62 | 0.84 | - |
| | **174** | - | - | 0.13 | 0.02 | - | 0.17 | 0.21 | - | - |
| | **176** | 0.03 | - | - | - | - | - | - | - | - |
| | **182** | - | - | - | 0.01 | - | - | - | - | - |
| | **184** | 0.03 | 0.07 | - | 0.13 | 0.20 | 0.20 | 0.17 | 0.05 | 0.50 |
| | **186** | - | - | - | 0.03 | 0.02 | - | - | - | 0.17 |
| **TmaA04** | **172** | - | 0.15 | - | - | - | - | - | - | - |
| | **185** | - | - | - | - | - | - | - | 0.05 | - |
| | **197** | - | - | - | - | 0.02 | - | - | - | - |
| | **199** | - | - | - | - | - | 0.01 | 0.01 | - | - |
| | **200** | - | - | - | - | - | - | - | - | 0.25 |
| | **201** | 0.03 | - | 0.17 | 0.27 | 0.27 | 0.37 | 0.34 | 0.08 | - |
| | **202** | - | - | - | - | - | - | - | 0.05 | 0.25 |
| | **203** | 0.56 | 0.23 | 0.17 | 0.14 | 0.10 | 0.07 | 0.09 | 0.13 | 0.50 |
| | **205** | - | 0.12 | - | 0.02 | - | - | - | - | - |
| | **207** | - | 0.12 | 0.33 | 0.04 | 0.15 | - | - | 0.05 | - |
| | **209** | - | 0.04 | - | 0.14 | 0.10 | 0.14 | 0.09 | 0.05 | - |
| | **211** | - | - | - | - | 0.02 | - | - | 0.03 | - |
| | **212** | - | - | - | - | - | - | - | 0.10 | - |
| | **213** | - | - | - | - | - | - | - | 0.13 | - |
| | **214** | - | - | - | - | - | - | - | 0.05 | - |
| | **215** | 0.41 | 0.19 | 0.17 | 0.18 | 0.21 | 0.01 | 0.02 | 0.03 | - |
| | **216** | - | - | - | - | - | 0.01 | - | - | - |
| | **217** | - | 0.08 | - | 0.03 | - | - | - | - | - |
| | **218** | - | - | - | - | - | - | - | - | - |
| | **219** | - | - | - | 0.01 | - | 0.01 | - | - | - |
| | **221** | - | - | - | 0.06 | 0.06 | 0.31 | 0.28 | 0.03 | - |
| | **223** | - | - | 0.17 | 0.03 | - | 0.03 | 0.05 | 0.05 | - |
| | **224** | - | 0.04 | - | 0.07 | 0.06 | 0.03 | 0.12 | 0.17 | - |
| | **225** | - | - | - | - | - | - | 0.01 | - | - |
| | **226** | - | 0.04 | - | - | - | - | - | - | - |
| | **228** | - | - | - | - | - | - | - | 0.03 | - |
| **TmaM79** | **143** | - | - | - | - | - | - | 0.01 | - | - |
| | **147** | - | - | - | 0.01 | - | - | - | - | - |
| | **149** | - | - | - | 0.05 | 0.05 | 0.01 | 0.01 | 0.05 | - |
| | **151** | - | 0.03 | - | - | - | - | - | - | - |
| | **153** | - | 0.03 | - | 0.01 | - | - | - | - | - |
| | **157** | - | - | - | 0.02 | - | - | - | - | - |

**Appendix 7** continued over page.

**Appendix 7** continued.

| Locus | Allele | Shark Bay | North WA | Northern Territory | Torres Strait | North Qld | Central Qld | South Qld | ASIA | PACIFIC |
|---|---|---|---|---|---|---|---|---|---|---|
| **TmaM79** | **159** | - | - | - | - | - | - | 0.08 | - | - |
| | **161** | - | - | - | - | - | 0.01 | 0.04 | - | - |
| | **162** | - | - | - | - | 0.02 | - | - | - | - |
| | **163** | 0.27 | 0.32 | - | 0.17 | 0.17 | 0.50 | 0.75 | 0.29 | - |
| | **164** | - | - | - | - | - | - | - | 0.05 | - |
| | **165** | 0.50 | 0.53 | 0.83 | 0.42 | 0.37 | 0.35 | 0.08 | 0.48 | 0.67 |
| | **167** | 0.23 | 0.11 | - | 0.11 | 0.15 | 0.06 | 0.02 | - | - |
| | **169** | - | - | - | 0.04 | 0.07 | - | - | 0.10 | - |
| | **170** | - | - | - | 0.01 | - | - | - | - | - |
| | **171** | - | - | 0.17 | 0.16 | 0.18 | 0.01 | - | - | 0.33 |
| | **179** | - | - | - | - | - | 0.03 | - | - | - |
| | **189** | - | - | - | - | - | 0.03 | - | 0.05 | - |
| **TmaE26** | **158** | - | - | - | - | - | - | 0.01 | - | - |
| | **160** | - | 0.05 | - | - | - | - | - | - | - |
| | **161** | - | - | - | - | - | - | 0.01 | - | - |
| | **162** | - | - | - | - | - | - | 0.01 | - | - |
| | **164** | 0.03 | - | - | - | - | - | - | - | - |
| | **166** | 0.05 | - | - | - | - | - | - | - | - |
| | **168** | - | - | - | 0.01 | - | - | - | - | - |
| | **170** | - | - | - | 0.01 | - | - | 0.01 | - | - |
| | **172** | - | - | - | 0.01 | - | - | 0.01 | - | - |
| | **174** | - | - | - | - | - | - | 0.01 | - | - |
| | **176** | - | - | - | 0.02 | - | - | - | - | 0.25 |
| | **178** | - | - | - | 0.04 | - | - | 0.01 | 0.02 | - |
| | **180** | 0.93 | 0.77 | 0.50 | 0.42 | 0.66 | 0.59 | 0.53 | 0.74 | 0.25 |
| | **182** | - | 0.10 | 0.50 | 0.41 | 0.34 | 0.39 | 0.37 | 0.22 | 0.50 |
| | **184** | - | 0.05 | - | 0.06 | - | 0.02 | 0.01 | 0.02 | - |
| | **186** | - | 0.03 | - | 0.01 | - | - | 0.02 | - | - |
| | **204** | - | - | - | 0.01 | - | - | - | - | - |
| **TmaA01** | **103** | - | - | - | - | - | - | 0.01 | - | - |
| | **109** | 0.43 | 0.36 | 0.63 | 0.75 | 0.57 | 0.64 | 0.46 | 0.74 | 1.00 |
| | **111** | 0.50 | 0.59 | 0.38 | 0.23 | 0.43 | 0.34 | 0.52 | 0.26 | - |
| | **113** | 0.07 | 0.05 | - | 0.01 | - | 0.01 | 0.02 | - | - |
| | **117** | - | - | - | - | - | - | - | - | - |
| | **119** | - | - | - | - | - | - | - | - | - |
| **TmaA02** | **210** | - | - | - | 0.01 | - | - | - | - | - |
| | **212** | - | - | - | 0.01 | - | - | - | - | - |
| | **232** | - | - | - | - | - | - | 0.01 | - | - |
| | **235** | - | - | - | 0.02 | - | - | - | 0.05 | - |
| | **236** | - | 0.18 | - | 0.25 | 0.37 | 0.12 | 0.08 | 0.18 | - |
| | **238** | 0.79 | 0.18 | 0.33 | 0.07 | 0.09 | 0.24 | 0.17 | - | - |

**Appendix 7** continued over page.

**Appendix 7** continued.

| Locus | Allele | Shark Bay | North WA | Northern Territory | Torres Strait | North Qld | Central Qld | South Qld | ASIA | PACIFIC |
|---|---|---|---|---|---|---|---|---|---|---|
| **TmaA02** | **239** | - | 0.07 | - | - | - | - | - | 0.05 | - |
| | **240** | 0.04 | 0.36 | 0.17 | 0.33 | 0.24 | 0.24 | 0.33 | 0.55 | 0.75 |
| | **244** | - | - | - | - | - | - | 0.01 | - | - |
| | **246** | - | - | 0.17 | 0.01 | - | - | - | - | - |
| | **247** | - | - | - | - | - | - | 0.01 | - | - |
| | **248** | - | - | - | 0.04 | - | - | 0.01 | - | - |
| | **249** | - | - | - | 0.01 | - | - | - | - | - |
| | **250** | - | 0.04 | - | 0.14 | 0.20 | 0.32 | 0.31 | - | - |
| | **252** | - | - | - | - | - | 0.05 | 0.04 | - | - |
| | **254** | - | 0.14 | - | - | - | - | - | 0.09 | - |
| | **256** | - | - | - | 0.01 | - | - | 0.01 | - | - |
| | **258** | - | - | - | 0.04 | 0.04 | 0.03 | 0.02 | - | - |
| | **260** | - | - | - | - | - | - | - | - | 0.25 |
| | **261** | 0.08 | - | - | - | - | - | - | - | - |
| | **262** | - | 0.04 | 0.17 | 0.02 | - | - | - | - | - |
| | **263** | 0.08 | - | - | 0.01 | - | - | - | - | - |
| | **264** | - | - | 0.17 | 0.04 | - | - | - | 0.09 | - |
| | **265** | - | - | - | 0.01 | 0.04 | - | - | - | - |
| | **266** | - | - | - | - | 0.02 | - | - | - | - |