Interspecific Hybridization in *Acropora* (Cnidaria: Scleractinia): Mechanisms and Evolutionary Consequences

Thesis submitted by
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Abstract

This thesis addresses several aspects of the genetics and reproductive biology of cross-fertile, mass-spawning scleractinian corals, specifically in the genus *Acropora*, and the results presented contribute to our understanding of the evolutionary consequences of hybridization in this animal group.

The rDNA ITS1-5.8S-ITS2 region has been used for phylogenetic analysis of cross-fertile coral species in the genus *Acropora*, and has shown patterns of variation consistent with reticulate evolution. However, results from a number of analyses in this thesis, including the occurrence of deamination-like substitutions at methylation sites; differences in evolutionary rates among clades of a 5.8S phylogeny; and ocurrence of non-compensatory mutations that may affect the rRNA secondary structure, suggest that at least part of rDNA diversity in *Acropora* is due to pseudogenes.

Natural hybridization in coral genera may cause taxa to merge through homogenization of gene pools or may create new hybrid species. Here I demonstrate that high cross-fertilization *in vitro* does not guarantee the merging of species. Data from eight polymorphic allozyme loci indicate small but significant differentiation between sympatric populations of *A. cytherea* and *A. hyacinthus*, a pair of acroporid corals with very high interspecific fertilization rates *in vitro*. The biological significance of differences between the species in sympatry is highlighted by the absence of genetic differentiation between widely allopatric populations within each species. Moreover, a Nested Clade Analysis using sequence data from a nuclear intron indicates that these two species constitute distinct evolutionary lineages. I conclude that *A. cytherea* and *A. hyacinthus* are neither merging nor constitute morphs within a single species, but rather conform distinct cohesion species.

Cross-fertilization trials may overestimate the rate of hybridization that occurs under natural conditions, because they are non-competitive, involving the exclusive combination of sperm from one species with eggs from another. I designed breeding trials using acroporid corals to test whether the mixture of conspecific and heterospecific sperm inhibits interspecific fertilization, promoting conspecific sperm precendence. However, spawning failure and low cross-

fertilization rate between the study species did not allow evaluating this hypothesis properly.

Integrins are proteins involved in cell adhesion that play major roles in gamete binding and fusion in mammals. A cDNA sequence encoding for a β1-class integrin has been identified in the scleractinian coral *Acropora millepora.* Given that the integrin mRNA is present in unfertilized eggs, the corresponding protein may have a potential role in coral fertilization. As a first attempt to elucidate the molecular basis of gamete specificity in corals, I studied the role of the *Acropora millepora* β_{Cn1} integrin in fertilization. I examined the effect of polyclonal antiserum raised against a substantial part of the β_{Cn1} integrin on fertilization rates of *A. millepora* eggs. The results indicate that *Acropora* β_{Cn1} integrin is involved in sperm-egg binding but does not confer reproductive specificity. The implication of a disintegrin-integrin binding in the fertilization process in *Acropora* suggests that some functions of these molecules may have been conserved in corals and humans.

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Chapter 1 General Introduction

Interspecific hybridization has always been a controversial topic in evolutionary biology, mainly due to conflicting interpretations about its evolutionary role. Botanists acknowledge the widespread occurrence of plant hybridization leading to reticulate patterns in plant evolution and highlight its creative role as source of new species (e.g. Stebbins 1959; Arnold 1997). On the other hand, zoologists have traditionally considered hybridization a rarity and a maladaptation for the species involved, reflecting imperfect reproductive barriers and thus an evolutionary dead end (reviewed in Harrison 1993; and in Dowling and Secor 1997). Such attitudes stem from the belief that hybridization disrupts coadapted gene complexes yielding inferior progeny, which leads to the reinforcement of reproductive isolation (Dowling and Secor 1997). Although detailed genetic studies on *Drosophila* have elucidated the basis of hybrid inviability/sterility (Coyne 1992; Wu and Palopoli 1994), the evidence for reinforcement of reproductive isolation has recently been questioned (Butlin 1987, 1989) and it has been shown that reinforcement only occurs under specific conditions (Kelly and Noor 1996). Several recent studies show that hybridization in animals is more common than previously thought and suggest that it may be responsible for both diversification and extinction (e.g. Vyas et al. 1990; DeMarais et al. 1992; Bullini 1994; Dufresne & Hebert 1994; Schartl et al. 1995; Carmona et al. 1997; Grant & Grant 1998; Parris 1999; Vila & Wayne 1999). This thesis addresses several aspects of the genetics and reproductive biology of cross-fertile, mass-spawning scleractinian corals, specifically in the genus *Acropora*, and the results presented contribute to our understanding of the evolutionary consequences of hybridization in this animal group.

Approaches to the study of natural hybridization

The difference in perception of the role of natural hybridization between botanist and zoologist partly stems from the use of distinct approaches to study hybridization. Botanist have traditionally followed the phylogenetic or 'pattern

oriented' approach, whereas the zoologist have focused on the 'hybrid zone framework' or 'process oriented' approach (Harrison 1993).

The phylogenetic approach simply involves the use of phylogenetic reconstruction to test for reticulation (reviewed in Arnold 1997). This methodology is particularly powerful when multiple data sets of different genetic markers are assayed. For example, this approach has been elegantly used to demonstrate homoploid hybrid speciation in sunflowers (Rieseberg et al. 1990; Rieseberg 1991).

Four major models have been defined within the hybrid zone framework. The Tension Zone model refers to hybrid zones as genetic clines maintained by a balance between dispersal and selection against hybrids (Barton and Hewitt 1985). This model is not always applicable because sometimes hybrid zones are mosaics of genotype frequencies, instead of being smooth transitions between alternate forms. This observation has led to the Mosaic model, which postulates that a hybrid zone arises from adaptation of the two parental species to different, patchily distributed environments (Howard 1986). Both the Tension Zone and the Mosaic models assume that hybrids are less fit than parentals. Alternatively, the Bounded Hybrid Superiority model proposes that hybrids are more fit in certain habitats (Moore 1977). However, the term "Bounded" was used to indicate that hybrid zones are usually narrow and located in ecotonal regions. Incorporating elements from all these models and based on evidence from field studies, Arnold (1997) proposed the "Evolutionary Novelty model". This latter model considers a) rarity of F1 hybrid formation, b) endogenous selection against certain hybrid genotypes, and c) exogenous selection acting against different hybrid genotypes, leading to d) the invasion of parental or novel habitats by more fit hybrid individuals. This model predicts the establishment of geologically long-lived, evolutionary lineages.

Although both a pattern oriented and a process oriented approach have been considered exclusive from one another (e.g. Nieto Feliner and Aguilar 1998), they are complementary, and both are required for a full understanding of the short and long term consequences of natural hybridization (Ritchie and Barton 1998). In this thesis both pattern and process oriented approaches were used to elucidate the consequences of hybridization on a pair of corals from the genus *Acropora* that exhibit high cross-fertilization rates *in vitro.* One major problem of

using a 'process oriented' approach to establish the consequences of hybridization in corals is that, given their long dispersal capacities, there are no well defined coral hybrid zones and many cross fertile species can co-occur along their whole distribution ranges. I therefore propose to use a comparative population genetic approach. Sympatric populations of two species that hybridize frequently (and are merging) would be expected to be more similar than allopatric populations within each species. Conversely, infrequent hybridization is predicted to lead to greater allele frequency differences between species in sympatry than between allopatric populations within species. These populations should also be compared with populations of a “control” species with known lower breeding compatibilities to establish a meaningful range of genetic distances that correspond with either isolation or interbreeding. In Chapter 3 I apply this approach to the study of hybridization between *A. cytherea* and *A. hyacinthus*.

Hybridization and reticulate evolution in scleractinian corals

Despite the fact that corals are ‘primitive’ metazoans, they have evolved via complex evolutionary pathways. Coral evolution has been affected by the capacity of these organisms for rapid, long-distance dispersal and the immense longevity of families, genera and species (Veron 1995). For example, long-distance dispersal limits allopatric speciation (Palumbi 1992) and, when it occurs, it may promote hybridization by secondary contact (Veron 1995). Moreover, most reef building corals breed in highly synchronized spawning events (Harrison et al. 1984; Willis et al. 1985; Babcock et al. 1986; Hayashibara et al. 1993; van Veghel 1993; Sanchez et al. 1999), creating opportunities for natural hybridization that are unparalleled in the animal kingdom (Babcock 1995) due to generally high interspecific cross-fertility (Miller and Babcock 1997; Willis et al. 1997; Szmant et al. 1997; Hatta et al. 1999). Taking into account these traits of corals, which resemble the life histories of plants more than those of animals, Veron (1995) postulated that the reticulate evolution model of plants is appropriate for corals. According to Veron (1995), changes in surface circulation in response to successive paleoclimatic cycles

drive cycles of contact and isolation among corals resulting in reticulate, rather than bifurcating, patterns of genetic connectivity at all systematic levels.

The reticulate evolution model predicts that natural hybridization in coral genera may cause taxa to merge through homogenization of gene pools or may create new hybrid species (Willis et al. 1997). The small number of species and very low genetic distances between mass spawning and cross-fertile species in the genus *Platygyra* (Miller and Benzie 1997; Willis et al. 1997) suggest that hybridization has led to homogenization of gene pools in this genus. Cross-fertilization also occurs among three members of the *Montastrea annularis* complex (Szmant et al. 1997), but both the taxonomic status and the role of hybridization are unclear in this group (i.e. Lopez et al. 1999; Medina et al. 1999). Very high rates of interspecific fertilization between Indo-Pacific species of *Acropora* (Willis et al. 1997; Hatta et al. 1999), the most species-rich genus of corals worldwide comprising between 113 (Wallace 1999) and 180 species (Veron 2000), suggest that hybridization may be responsible for the formation of new species in this genus (Willis et al. 1997). The large number of *Acropora* species in the Indo-Pacific complicates unravelling phylogenetic relationships, but genetic evidence suggests a hybrid origin for at least one species in the simpler three-species system of the Caribbean (van Oppen et al. 2000; S.V. Vollmer unpublished data). However, it is possible that hybridization among *Acropora* species causes the creation of new species in some cases but leads to homogenization in others.

The genus *Acropora* as a system to study coral evolution and the species selected for this study

This study constitutes part of a multidisciplinary approach to examine species boundaries of cross-fertile *Acropora* corals and to understand the evolutionary consequences of interspecific hybridization in this genus (Wallace and Willis 1994; Willis et al. 1997; van Oppen et al. 2000, 2001a, submitted). The focus on the *Acropora* is justified by its ecological importance, since it is the most widespread, abundant and species-rich genus of reef-building corals (Wallace 1999). In addition, *Acropora* has the practical advantage that its reproduction is predictable (Willis et al. 1985), external and accessible and that the base

content of its genome is not strongly biased (Miller and Ball 2000). Moreover, the taxonomy of this genus is very challenging, due to the morphological variability found within many *Acropora* species as well as the morphological similarity that occurs between some species (Wallace and Willis 1994).

The species *Acropora hyacinthus* and *A. cytherea* are the focus of this thesis (Figure 1.1), because they have one of the highest interspecific fertilization rates recorded (mean=50%) (Willis et al. 1997). Therefore they constitute an upper limit for the known possible range of hybridization within the genus. Both species grow as flat-topped tables and occur at most reef locations throughout the Indo-Pacific (Wallace 1999). They can be distinguished on the basis of corallite arrangement, which is rosette-like in *A. hyacinthus* but scale-like in *A. cytherea*. However, this distinction can vary geographically, making it relatively easy to separate both species on the Great Barrier Reef (GBR) but not so in the Central Pacific (Veron 1995, p. 285). There is also a slight ecological differentiation between these species along a depth gradient, with *A. cytherea* typically occurring deeper than *A. hyacinthus*.

Chromosome numbers and molecular phylogenies agree with the reticulate evolution model

A study of chromosome numbers in 22 species of *Acropora* indicated that most have 2n=28 chromosomes, except for six species which have somatic chromosome numbers of 24, 30, 30, 42, 48 and 54 (Kenyon 1997). According to Kenyon, this pattern could be explained by subsequent hybridization events that generated polyplody and aneuploidy (i. e. reduction or increase in chromosome number). Polyploid speciation can be rapid and can occur in sympatry, possibly accounting for the large number of species within the genus *Acropora* (Kenyon 1997).

Results from molecular systematics on corals are also congruent with the reticulate evolution model. For example, several species of *Acropora* share different types of ribosomal DNA (Odorico and Miller 1997; van Oppen et al. 2000; van Oppen et al. submitted), and the same happens in the genus *Madracis* (Diekmann et al. 2001). Additionally, species-level molecular phylogenies using both mtDNA and single copy nuclear markers in *Acropora*

show extended para- and polyphyly (Hatta et al. 1999; Fukami et al. 2000; van Oppen et al. 2001a). Nevertheless, these patterns of allele and haplotype sharing may also be interpreted as the result of recent speciation and retention of ancestral polymorphisms (van Oppen et al. 2001a; Diekmann et al. 2001). Moreover, the use of rDNA for phylogenetic analysis of hybridizing taxa can lead to an overestimation of the amount of introgression (i.e. the interspecific exchange of genes that follows hybridization). If introgressed rDNA types are relocated in different chromosomes, the homogenizing action of concerted evolution can be severely reduced (Arnheim et al 1980) and the different copies can be maintained in hybrids and their descendants. Alternatively, nucleolar dominance can cause the silencing of some of these rDNA types (Honjo and Reeder 1973; Durica and Krider 1977), which can then mutate freely and become pseudogenes (Muir et al. 2001). Chapter 2 of this thesis addresses whether some of the highly divergent rDNA types shared among *Acropora* species are indeed pseudogenes.

Species concepts in hybridizing corals

Coral taxonomy is primarily based on skeletal characters, which exhibit considerable variation across individuals, populations and species (Veron 1981; Knowlton and Weigt 1997). Species boundaries are consequently fuzzy and arbitrary (Veron 1995). The discoveries in breeding compatibilities and molecular biology mentioned above are challenging the application of the morphological species concept in coral taxonomy (Wallace and Willis 1994). The Biological Species Concept (BSC) states that "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Dobzhansky 1937). Therefore, significant levels of hybridization invalidate the application of the BSC to corals. Among many other problems, derivatives of the BSC also pose difficulties concerning hybridizing species because according to these, hybridizing morphospecies should be considered races of one species. These concepts are: the Recognition Species Concept that defines species as the "most inclusive population of individual biparental organisms which share a common fertilization system" (Paterson 1985); the Evolutionary Species Concept, where

species are defined as populations, or groups of populations that share a common evolutionary history and are held together by developmental, genetic and ecological constrains (Simpson 1944); and the Phylogenetic Species Concepts under which species are "an irreductible (basal) cluster of organisms, diagnosably distinct from other such clusters and within which there is a parental pattern of ancestry" (Cracraft 1983). Unlike the above, the cohesion species concept (CSC) (Templeton 1989) accommodates limited hybridization, and its application can potentially resolve shared ancestral polymorphisms / incomplete lineage sorting from hybridization using objective and quantifiable criteria (Templeton 2001). The cohesion species is a population of organisms that constitute both a distinct evolutionary lineage as well as a reproductive community in either a genetic or adaptational/ecological sense (Templeton 1989, 1994). Chapter 4 describes the application of the cohesion species concept to *Acropora hyacinthus* and *A. cytherea*.

Interspecific sperm competition as a mechanism to maintain cross-fertile coral species as distinct genetic entities

The likelihood of occurrence of natural hybridization in corals has only been partially addressed. Experimental trials have shown that interspecific fertilization occurs *in vitro* and is feasible *in vivo* (Miller and Babcock 1997; Szmant et al. 1997; Willis *et al.* 1997). However, as in many other studies of hybridizing species, these may have overestimated the rates of hybridization that actually occur under natural conditions, because gamete competition was not considered (reviewed in Arnold 1997). *In vitro* cross-fertilization trials indicate high breeding compatibilities among several coral species, but these experiments generally involve the exclusive combination of sperm from one species with eggs from another (Miller and Babcock 1997; Szmant et al. 1997; Willis et al. 1997; Hatta et al. 1999). In contrast, during mass spawning events eggs are likely to be exposed to complex mixtures of conspecific and heterospecific sperm, allowing gamete competition to potentially reduce levels of interspecific fertilization.

Chapter 5 describes the use of mixtures of hetero- and conspecific sperm in breeding trials in attempts to test whether conspecific sperm takes precedence.

The molecular basis of gamete specificity in *Acropora*

In vitro hybridization in scleractinian corals occurs most readily between species that are morphologically similar (Willis et al. 1997). Therefore, some level of specificity in the fertilization process must exist. Recent advances in understanding the specificity of gamete interactions in abalone and sea urchins have shown that particular sperm proteins interact with egg proteins in a species-specific manner (e.g. Gao et al. 1986; Vacquier 1998). Homologous genes may operate in corals, because they are also marine invertebrates with external fertilization. However, as discussed in chapter 6 this appears to be extremely unlikely. As an alternative, I explore the role of an integrin identified in *Acropora millepora* (β_{Cn1}) (Brower et al. 1997) in fertilization. Integrins are proteins involved in cell adhesion and play major roles in gamete binding and fusion in mammals (reviewed in Bowen and Hunt 2000). Given that β_{Cn1} integrin mRNA is present in unfertilized coral eggs, the corresponding protein may have a potential role in coral fertilization.

Aims of this thesis

- To reassess the phylogenetic utility of the ITS-5.8S region of the rDNA in *Acropora*, determining whether some of the types are indeed pseudogenes (**Chapter 2**).

- To determine whether *A. hyacinthus* and *A. cytherea* are hybridizing on a frequent basis, by establishing whether sympatric populations of both species are genetically more similar to each other than to allopatric populations within each species (**Chapter 3**).

- To determine whether *A. hyacinthus* and *A. cytherea* conform to the phylogenetic and/or cohesion species concepts, by testing whether they are monophyletic in molecular phylogenies; and/or constitute distinct evolutionary lineages according to Nested Clade Analysis (**Chapter 4**).

- To determine whether interspecific sperm competition in *Acropora* results in conspecific sperm precedence (**Chapter 5**).

- To contribute to the elucidation of the molecular basis of the limited gamete specificity in corals, by establishing the role of the *Acropora millepora* β_{Cn1} integrin in fertilization (**Chapter 6**).

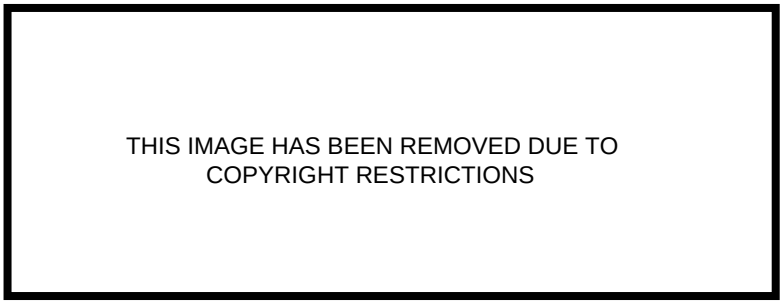

Figure 1.1 General colony morphology (a) and detail of corallites of *Acropora hyacinthus* (b) and *A. cytherea* (c) (From Wallace 1999).

Chapter 2 The origin of extreme nuclear ribosomal DNA diversity in *Acropora*

Abstract

The rDNA ITS1-5.8S-ITS2 region has been used for phylogenetic analysis of cross-fertile coral species in the genus *Acropora*, and has shown patterns of variation consistent with reticulate evolution. However, a recent study in oaks indicates that divergent rDNA copies shared among hybridizing species can be pseudogenes that predate speciation. To evaluate the phylogenetic value of ITS regions in *Acropora* and determine whether some of the variants are indeed pseudogenes, I expanded the range of species, number of samples and sampling localities as compared to the original studies of rDNA variation. Results from a number of analyses, specifically phylogenetic analysis using maximum likelihood of 5.8S rDNA sequence data (because ITS1 and ITS2 are too variable and probably saturated), a recombination analysis, a relative-rate test, a mutation spectrum analysis were compared. Although not completely congruent, major features of the 5.8S phylogeny corresponded with phylogenies generated using DNA sequences of a single copy nuclear intron and an intergenic region of the mtDNA. The sharing of 5.8S sequences between different species and the non-monophyly of species may be the signature of either introgression or ancestral polymorphism. Most 5.8S sequences cluster in a single clade (IV). A derived subclade (IVC) within this large clade is composed mainly of clones of specimens that are also represented in a larger basal subclade (IVB). Members of this derived subclade (IVC) have both a higher evolutionary rate and a higher number of deamination-like substitutions per sequence at methylation sites as compared to the basal subclade (IVB). Moreover, the sequences from subclade IVC share non-compensatory mutations that can potentially disrupt the secondary structure of the large subunit of the rRNA. Based on these and preliminary results from an expression analysis, indicating that the expressed rDNA types correspond to those of subclade IVB, I conclude that the derived subclade IVC represents pseudogenes.

Introduction

One of the first markers employed to study reticulate evolution in acroporid corals was the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal RNA (rRNA) transcription unit (the rDNA) (Odorico and Miller 1997). rDNA constitutes a multigene family of tandem repeats, that in contrast to single copy genes do not evolve independently, but in a concerted manner (Arnheim et al. 1980). This phenomenon has been called concerted evolution and the underlying molecular processes are unequal crossing over and gene conversion (Dover 1982). As a result of concerted evolution, the rDNA families are usually homogeneous within individuals and species, but interspecific divergence can be high (Hillis and Dixon 1991). Moreover, sequence types of the parental species are additively combined in hybrids, making rDNA the most frequently used genetic marker in studies of hybridization and introgression (e.g. Sang et al. 1995; Quijada et al. 1997; Brasier et al. 1999).

Odorico and Miller (1997) found very high levels of variability in both ITS1 and ITS2 within and among several species of *Acropora*. Most interestingly, the studied species shared several distinct ITS2 types, and a putative intermediate of two types present in *A. hyacinthus* and *A. cytherea* was found in a colony of the latter, which was consistent with the high *in vitro* cross-fertilization rates observed for these species (Willis et al. 1997). Therefore, these results were interpreted as evidence for recent interspecific hybridization, supporting the reticulate evolution hypothesis. In the *Acropora aspera* group, all of the five species studied share rDNA types (van Oppen et al. submitted). However, most *A. aspera* sequences constitute a distinct clade in phylogenetic analyses. This result is consistent with the observations of a semi-permeable temporal barrier involving differences in spawning times in some years between *A. aspera* and the other four species studied by van Oppen et al. (submitted). In the three Caribbean *Acropora* species, similar rDNA sequences were shared among species (van Oppen et al. 2000). The level of variability of the ITS region in the Caribbean *Acropora* species was smaller than that found among Indo-Pacific species of *Acropora*, and this was explained by arguing a smaller Caribbean 'syngameon', i.e., ‘‘the sum total of species or semispecies linked by frequent or occasional hybridization in nature; (hence) a hybridizing group of species ...’’(Grant 1957).

Nevertheless, even when the reticulate evolution scenario in *Acropora* has gained support by the results of phylogenetic analysis using both nuclear and mitochondrial markers (Hatta et al. 1999; Fukami et al. 2000; van Oppen et al. 2001a), there are alternative explanations to explain these results. Firstly, these patterns of allele and haplotype sharing may also be interpreted as the result of recent speciation and retention of ancestral polymorphisms (van Oppen et al. 2001a; Diekmann et al. 2001). Secondly, the use of rDNA for phylogenetic analysis of hybridizing taxa can lead to an overestimation of the amount of introgression. For example, when the rDNA copies are located at different chromosomal positions, the homogenizing action of concerted evolution can be severely reduced (Arnheim et al 1980) and the different copies are maintained in hybrids and their descendants. Nucleolar dominance can cause the silencing of some of these rDNA types (Honjo and Reeder 1973; Durica and Krider 1977), which can then mutate freely and become pseudogenes (Muir et al. 2001). It is believed that this may have happened in the hybridizing oak species *Quercus petrea* and *Q. robur*. These two species hybridize frequently and share three divergent rDNA types, two of which are believed to be pseudogenes whose origin predates the species divergence (Muir et al. 2001). Up to nine rDNA types can be present in a single *Acropora* colony, and each type can be represented by more than one sequence (Odorico and Miller 1997; van Oppen et al. submitted). Hence, it is possible that repeat units occur on more than one chromosome or chromosomal position and that some of the rDNA types shared among species represent pseudogenes.

In this chapter I analyze a large number of sequences of the 5.8S gene as well as the ITS2 region (for a subset of the sequences that were alignable in ITS2) from a broad range of *Acropora* species to: 1) compare the phylogeny based on the 5.8S gene with those obtained using a single copy nuclear intron and an intergenic region of the mtDNA, i.e. to estimate the phylogenetic value of the rDNA; 2) investigate the spatial variation in the distribution of rDNA types, arising from different distribution ranges of hybridizing species; 3) estimate the amount of recombination in these sequences and determine whether it corresponds with the breeding compatibility data (i.e. species with high compatibility show a high recombination signal); and 4) determine whether

some of the rDNA types shared among species are possible candidates for pseudogenes.

Materials and Methods

Sample collection

Table 2.1 indicates the species designation and geographic origin corresponding to each sequence newly obtained. I also included Odorico and Miller's (1997) data set, some Caribbean representatives (*A. cervicornis* group) (van Oppen et al. 2000), sequences from the *A. aspera* group (van Oppen et al. submitted) and from the *A. nasuta* group (Mackenzie in prep.) in the analysis. Tissue samples were collected by snapping off small branches (2-5 cm) from individual colonies and storing them in 70-90 % EtOH.

Laboratory Techniques

DNA extraction, PCR and cloning procedures followed van Oppen et al. (1999), except that different PCR and sequencing primers were used. For PCR the primers were Acf (5'-ACCGATCGAACGGTTTAG-3') and Acr (5'-ACGCTCCCTTCCAAGAGA-3'). These are *Acropora*-specific and anneal to the ssu and lsu rDNA genes respectively. For sequencing, the internal ssu primer A18f (5'-GAACTTGATCGTTTAGAG-3') and the lsu internal primer A28r (5'-CTGGTTAGTTTCTCGTCC-3') were used. PCR products were cloned into pGEM-T (Promega) following the manufacturer's protocol. Positive clones were amplified by colony PCR, purified and sequenced on an ABI 310 Genetic Analyzer as described in van Oppen et al. (1999).

Alignment

Sequences were aligned manually using Sequencher 3.0 (Gene Codes Corporation). The gene/spacer boundaries were identified using Odorico and Miller's (Odorico and Miller 1997) alignment of *Acropora* rDNA sequences. The 5.8S region was easily alignable, but even following the Odorico and Miller's alignment, it was practically impossible to align the ITS1 and ITS2 regions

objectively. Of both ITS1 and ITS2, the former was the most difficult to align (as has been observed before, Odorico and Miller 1997) and therefore was not used in subsequent analyses.

Phylogenetic Analyses

Due to the difficulty in aligning the ITS regions, only the 5.8S gene was used for the phylogenetic analyses across the entire data set. Excluding identical copies and leaving only those differing in at least one position, the data set was reduced from 416 to 160 sequences. A maximum likelihood phylogeny was constructed using the HKY85 (Hasegawa et al. 1985) model of molecular evolution in MOLPHY 2.3 (Adachi and Hasegawa 1996). The tree was rooted using *Acropora* (*Isopora*) *cuneata* as an outgroup. The subgenus *A. Isopora* forms a sister group to *A. Acropora* in both cladistic analyses using morphology (Wallace 1999) and sequence analysis of Cytochrome b and NADH dehydrogenase subunit 2 (van Oppen et al 1999; Fukami et al. 2000). Analysis of molecular variance (AMOVA) (Excoffier et al 1992) was used to partition the genetic variance amongst species and amongst the phylogenetic clades using Kimura two-parameter distance (1980) in ARLEQUIN 2.0 (Schneider et al. 2000).

A permutation test for the existence of phylogenetic signal [Permutation Tail Probability (PTP)] in the 5.8S data set was performed generating 100,000 random trees and comparing the lengths of these with a consensus one obtained by parsymony analysis in Paup 4.0 beta (Swofford 1999). The skewness in the distribution of the lengths of another 100,000 randomly generated trees was also used to evaluate the phylogenetic signal of this data set (i. e. strong phylogenetic signal is characterized by a left skew in the tree length distribution, caused by very few trees having short lengths).

ITS1 was unalignable, however ITS2 was relatively easy to align in a subset of 185 sequences corresponding to the largest clade in the 5.8S phylogeny (clade IVB, see below). Phylogenetic analysis of ITS2 was only applied to this subset of sequences.

Recombination

Recombination signatures were visually examined with "Phylogenetic Profiles" using the program PhylPro (Beta) 0.81 (Weiller 1998). Using a sliding-window technique, this method determines pairwise distances between all sequences within the window and evaluates, for each sequence, the degree to which the patterns in contiguous windows agree. Disagreement between the patterns is expressed as a correlation coefficient, where low values represent strong disagreement indicating the potential signature of a recombination event. This analysis was performed only for the 5.8S and ITS2 alignments used in the phylogenetic analyses, due to the unreliability of the alignments of ITS1 or the whole ITS2 data set that would overestimate the recombination signal.

Relative-Rate Test

A two-cluster relative-rate test (Takezaki et al. 1995) using Kimura two-parameter distance (1980) was implemented in Phyltest 2.0 (Kumar 1996) to examine the evolutionary rate constancy of rDNA genes among the resulting clades in the phylogeny. This test examines the constancy of molecular clock for two lineages when an outgroup lineage is given. If L_a and L_b are the averages of observed numbers of substitutions per site (branch lengths) from the common ancestor of clusters A and B, then $L_a = L_b$ is the null hypothesis under the constancy of molecular clock, *i.e.*, $d = L_a - L_b = 0$. Because the variance of d can be estimated, we can test the deviation of d from 0 (and thus constancy of evolutionary rates between lineages A and B) by a two-tailed normal deviate test.

A representative of each clade (from Figure 2.1) was included in the analysis. The test was only performed for the 5.8S region.

Methylation-Related Substitutions

DNA methylation is suspected to function as a silencing mechanism in nucleolar dominance, thus putative pseudogenes are expected to contain elevated numbers of deamination-like substitutions (C→T and G→A). Therefore, following Muir et al. (2001) and using *A. cuneata* as a reference for the ancestral state, I counted the number of cytosine sites showing at least one

deamination-like substitution and those that did not contain any of such substitutions within each clade. I then compared these numbers for each clade with those of the most basal clade in the phylogeny, assuming that it represents the ancestral state, using a 2 x 2 contingency table. Given the small sample sizes, the asymptotic property of a chi-square distribution cannot be assumed. Instead, I performed an exact test that uses the random permutation procedure of Roff and Bentzen (1989). In this procedure, a contingency chi-square statistic is calculated and the probability of observing the exact test statistic or larger is generated using a random permutation procedure that maintains the marginals but simulates the null hypothesis of no association. The random permutation is implemented in Chiperm 1.2 (Chiperm, is available at the Crandall lab web site at http://bioag.byu.edu/zoology/crandall_lab/programs.htm).

Base substitutions at conserved sites

Pseudogenes are expected to mutate freely, as they are not under selective constraints. I identified conserved sites in the 5.8S based on the alignment of 30 sequences from a broad range of taxa, including fungi, plants, invertebrates and vertebrates, and then counted the number of mutations occurring at those conserved sites in sequences belonging to each clade obtained in the *Acropora* phylogeny.

Evaluation of secondary structure

The secondary structure of large subunit of the rRNA (that includes the 5.8S gene) is conserved among eukaryotes, even when some areas show considerable variability in length and base composition (Wuyts 2001). This conservation of the rRNA secondary structure is due to constraints imposed by its regulatory role in protein translation (Cech and Bass 1986). As with the analysis of mutation at conserved sites, it would be expected that candidates for pseudogenes would mutate freely and that some of these mutations would be non-compensatory, affecting the secondary structure of the rRNA. A set of a broad range of metazoan 5.8S sequences, containing annotations of the

secondary structure derived by comparative sequence analysis, was obtained from the European Large Subunit Ribosomal RNA Database (Wuyts et al. 2001) (http://rrna.uia.ac.be/lsu). I aligned representatives from each clade of my phylogeny with this data set to look for non-compensatory mutations that may disrupt the formation of helices.

Results

The complete data set consists of 416 sequences of ITS1-5.8S-ITS2 from a range of *Acropora* species. More than 60% of 5.8S sequences were repeated sequences (Table 2.2) and 147 of them were identical to *A. hyacinthus* Aht.1 in Odorico and Miller (1997). ITS sequences were in general very different from one another and sometimes almost impossible to align. The maximum length of these sequences (Table 2.3) was slightly larger than those reported previously [i.e. 166 and 200 for 5.8S and ITS2, respectively, as compared to 112 and 158 reported by Odorico and Miller (1997)], due to the inclusion of the *Acropora (Isopora) cuneata* sequences containing inserts not present in the subgenus *Acropora Acropora*. The base composition of both regions (Table 2.3) did not differ significantly among species, subgenera or in comparison to those previously reported (i.e. Odorico and Miller 1997).

Levels of genetic diversity in the 5.8S gene within the subgenus *Acropora* were extremely high, with a maximum corrected distance between *A. cerealis* and *A. gemmifera* of 20.3% (Table 2.3). The distance between subgenera was slightly higher, with a maximum of 22.8% between *A. cuneata* and *A. gemmifera*. Figure 2.1 shows the alignment of representatives from observed rDNA types, following Odorico and Miller (1997). ITS2 distances estimates were very high (Table 2.3) and the difficulty aligning these sequences suggest that they are saturated, although estimates of transition/transversion ratios were 0.95 and 1.01 for ITS1 and ITS2 respectively. Another possibility is that these rapidly evolving non-coding regions are simply too divergent.

Phylogenetic analysis

The 5.8S alignment of 160 sequences used in the phylogenetic analysis consisted of 166 positions of which 58 were constant, 33 were variable but parsimony uninformative and 75 were parsimony informative. The phylogenetic signal in this alignment was high, as indicated by the results of a permutation test (PTP) (the length of most parsimonious tree was 227 steps, whereas the shortest length of a randomly generated tree was 606, P = 0.01), and by a significantly left-skewed tree length distribution among 100,000 random trees (g_1= -0.419) (Hillis and Huelsenbeck 1992). The consistency (CI) and retention (RI) indices of 0.62 and 0.91, indicate a small level of homoplasy.

Four major clades were distinguished in the 5.8S phylogeny (Figure 2.2), which correspond to the most distinctive ITS2 types (Figure 2.1). The base of the tree is composed by three clades and is unresolved forming a polytomy. These basal clades are mainly composed by sequences from the *A. nasuta* and *A. aspera* group (Clade I); the Caribbean *A. cervicornis* group (Clade II); and the *A. selago* and *A. echinata* groups (Clade III). The derived clade IV is subdivided in five smaller subclades, mainly composed by sequences belonging to a few *Acropora* species. In particular, subclade IVA is composed mainly by *A. gemmifera* and *A. longicyathus*; subclade IVB by species from the *A. hyacinthus* and *A. aspera* groups; subclade IVC by *A. millepora*; subclade IVD by *A. aspera* and *A. florida*; and subclade IVE by *A. pulchra* and *A. papillare*. However, representatives of some species occur throughout the tree. Interestingly, all sequences from the Caribbean *A. cervicornis* group occur only in clade II. Note that clones from single individuals sometimes occur in completely different clades (e.g. *A. spicifera*-79 clones occur in subclades IIIA and IVB). This is also obvious from the AMOVA, which indicates that the percentage of variance explained by the difference between clades is higher than the one explained by the difference between species, and it is very high within species (Table 2.4).

The clades produced by a phylogenetic analysis using ITS2 sequences from the individuals represented in subclade IVB in the 5.8S phylogeny, which were the only ones easily alignable, do not correspond to either species or geographic localities (Figure 2.3).

Recombination

The phylogenetic profiles showed several recombination signatures in the 5.8S region (Figure 2.4A). None of the most recombinant sequences belonged to the *A. hyacinthus* group (Table 2.5). The small subset of ITS2 sequences did not show a very high recombination signal (Figure 2.4B). Unfortunately, given the difficulty aligning ITS2, an evaluation of recombination among types could not be performed. The recombination signal may be overestimated towards the edges of the graphs. This is due to a slight decrease in the accuracy of the Phylogenetic Correlation slightly in these regions caused by the reduction in size of one of the compared windows (upstream or downstream) when the point of comparison moves closer to the each edge (Weiller and Van den Borre 1998).

Relative-rate test

Rate constancy may differ between different groups of species, as they are not necessarily exposed to the same evolutionary constraints. On the other hand, it would be expected that within species the rate constancy should be similar (Muir et al. 2001). The results from the relative-rate test indicate that different clades and sub-clades in the 5.8S phylogeny have different constancy rates (rate constancy rejected at 5% level, Table 2.6). Clade I has a different rate in comparison to II and III, and IV is different from the other three. *A. millepora* and *A. pulchra* sequences are spread between subclades IVB, IVC and IVE, which have different rates. Different constancy rates among sequences from the same species (in this case even cloned from the same individual) suggest that some of these sequences may constitute pseudogenes.

Methylation mutation analysis

Without further analysis, it cannot be ruled out that rDNA copies with different evolutionary rates found within the same species or individual are functional but located in genomic regions exposed to an elevated mutation rate. Table 2.7 shows that the absolute number of deamination-like substitutions at methylation sites in the 5.8S (in comparison to the number of mutations at non-methylation

sites) was not significantly higher in any clade or subclade as compared to the outgroup (*A. cuneata*), which is considered to represent better the ancestral state. However, the number of deamination-like substitutions at methylation sites per sequence in subclade IVC was surprisingly high (21 in 14 sequences) and significantly larger than in subclade IVB (11 in 57 sequences), in relation to the number of substitutions in non-methylation sites (4 and 7 respectively) (chi-square=8.52, *P*=0.008). This evidence supports that subclade IVC could constitute pseudogenes.

Analysis of conserved sites

I found a total of 45 conserved positions amongst 30 different taxa, ranging from fungi to humans (Figure 2.5). Although all *Acropora* clades had mutations in at least one of those sites, the number of mutations was very small in all cases (Table 2.8). The highest number of mutations (5) was observed in the *A. cerealis* 114.1 sequence, present in clade IVC.

Evaluation of secondary structure

At least three sequences from clade IVC contained non-compensatory mutations (Figure 2.6). These mutations occur in helices B6 and B9 (Figure 2.7). The mutation in B6 and one (two in *A. cerealis* 114.1) in B9 would require the pairing of two guanine residues in order to maintain the secondary structure of these helices. This is extremely unlikely because there is insufficient room for two purines in the 10.8 Å space of the glycosidic bond.

Discussion

Characteristics of the rDNA region in Acropora

These results confirm the notion that the rDNA ITS regions of *Acropora*, including the subgenus *Isopora*, are amongst the shortest in corals (Takabayashi et al. 1998) and eukaryotes in general (Odorico and Miller 1997). The levels of variability in the 5.8S and ITS2 region of *Acropora*, up to 20.3 % and 59.5% respectively, are the highest reported for any coral genus. The

complete ITS1-5.8S-ITS2 region exhibits only 2% variation among morpho-species of the *Montastrea annularis* complex (Lopez and Knowlton 1997; Medina et al. 1999), and only up to 4.9 % between species of the genus *Madracis* (Diekmann et al. 2001). Takabayashi et al. (1998) reported only ITS1 variation, which was 15% in *Goniopora tenuidens*, 2% in *Heliofungia actiniformis*, 11% in *A. longicyathus* and 31% in *Stylophora pistillata*. In *Plesiastrea versipora*, a species with an extreme latitudinal range (40° N to 40°S) and accordingly expected to be genetically variable, populations across the whole range had variabilities of just 1.53% ± 0.43 and 3.15% ± 2.24 in ITS1 and ITS2, respectively (Rodriguez-Lanetty and Hoegh-Guldberg accepted). Similar to my study, in several species of *Porites*, Hunter et al. (1997) found high levels of variation in ITS1 and ITS2 (49% and 48% respectively), but lower variability in 5.8S (3%). Overall genetic differences between Hawaiian and Caribbean *Porites* species ranged up to 21%. Despite the 5.8S subunit being a coding region, I estimated corrected genetic divergences of approximately 20% within the subgenus *Acropora*, which is five times higher than the estimate of Odorico and Miller (1997) using a smaller sample size and almost twice as high as in the aspera group alone (van Oppen et al. submitted). These high levels of variability give rise to the question of whether some of these 5.8S genes have lost their functional constraints and are evolving as pseudogenes (see below).

Phylogeny

The topologies of *Acropora* phylogenies obtained so far (van Oppen et al. 2001a; Chapter 4) present several common features such as the derived and distinctive *A. aspera* clade with *A. florida* as a sister taxon, and the basal *A. tenuis* - *A. longicyathus* clade (van Oppen et al. 2001a; Chapter 4). The 5.8S phylogeny resembles that of the mtDNA in that the Caribbean taxa are amongst the basal groups. However, *A. aspera* is not monophyletic based on 5.8S and the base of the tree is unresolved forming a trichotomy between the clade containing the Caribbean taxa and A*. longicyathus/A. tenuis*, the *A. cerealis/A. millepora* clade and the remaining taxa. Incongruence between nuclear and mitochondrial acroporid phylogenies may reflect either introgression or recent speciation and incomplete lineage sorting (van Oppen et al. 2001a). Avise and

Ball (1990) showed that short separation times result in a lack of lineage sorting and genealogical concordance. This causes phylogenetic analyses of molecular markers to represent gene genealogies, rather than organismal phylogenies.

A key feature conserved between the 5.8S phylogeny and both the nuclear intron and mtDNA phylogenies presented in van Oppen et al. (2001), is the presence of sequences of the same species in more than one clade, even when these clades have very high bootstrap support and long branches. In the 5.8S tree there are even clones from the same individual in different clades. However, the sequences are not evenly distributed among clades, as most sequences of a single species occur in one clade. Also, some 5.8S types were shared among species, but also at very small frequencies. This observation of species present in more than one clade also corresponds with the AMOVA results, which indicate that the genetic variance is only slightly higher between than within species. These patterns reflect either recent speciation coupled with incomplete lineage sorting or introgression (van Oppen et al. 2001a). Nevertheless, some species clustered exclusively within a single clade, such as all the sequences of species in the Caribbean *A. cervicornis* group. This observation and the fact that individual species within this group were not monophyletic, support the hypothesis that they constitute a smaller and independent syngameon (i.e. hybridizing group of species) in comparison to the Indo-Pacific *Acropora* spp. (van Oppen et al. 2000).

Apart from the clustering of all the species in the *A. palmata* group, in which taxonomy and geographical location are correlated, there was no geographic signal in either the 5.8S or the ITS2 phylogenies.

Due to the extremely high levels of variability in ITS sequences, I do not recommend its use for phylogenetic analysis in *Acropora*. Although there are incongruences between the 5.8S phylogeny and the nuclear intron and mtDNA phylogenies (van Oppen et al. 2001a), the 5.8S gene seems to provide a high phylogenetic signal based on the permutation test and the left-skewed tree length distribution.

Recombination

The species showing the highest 5.8S recombination signal, *A. longicyathus*, exhibits extremely high genetic diversity, probably due to the presence of types

that cluster with either *A. tenuis* in clade III or with *A. gemmifera* in clade IV. It is possible that this pattern represents cryptic speciation, with some level of introgression between these taxa explaining the high recombination signal. Research into the breeding compatibility between *A. longicyathus* colonies and species from both clades may help us to understand these observations.

It was expected that sequences of *A. cytherea* and *A. hyacinthus* would present a high recombination signal, given that Odorico and Miller (1997) found a recombinant sequence in an *A. cytherea* and because these two species have one of the highest interspecific fertilization rates recorded from *in vitro* crosses (mean = 50%) (Willis et al. 1997). However, the phylogenetic profile did not indicate a higher recombination signal in these two particular species. This can be due simply to the relative representation of the recombination signal and the presence of even more recombinant sequences from other species (Weiller 1998). Notice that *A. cuneata* appears to present a high recombination signature.

Identification of pseudogenes

The relative rate test indicated that clades containing different groups of species had different substitution rates, which was expected as they are not necessarily exposed to the same evolutionary constraints. However, clade IVC was composed mainly of sequences from coral colonies that were also represented in other clades, suggesting that these particular clones could be pseudogenes. This notion is supported by the high number of actual substitutions per sequence at methylation sites in sub-clade IVC, which was significantly larger than in sub-clade IVB. The number of mutations at conserved sites was not higher in clade IVC, although *A. cerealis* 114.1, a member of that clade, had the highest number of mutations. Additionally, sequences from clade IVC shared non-compensatory mutations that could potentially disrupt the secondary structure of the large subunit of the rRNA. One of these mutations is believed to disrupt the conformation of helix B6, while at least two other mutations possibly affect the conformation of helix B9 that links the 5.8S with the 28S. I consider all this enough evidence to support that the rDNA copies of clade IVC constitute pseudogenes.

A preliminary study to identify the expressed and hence functional rDNA types in *A. millepora* produced five sequences obtained from cytoplasmic RNA that all clustered in clade IVB (D. de Jong et al., unpublished data). Although more sequences are needed before reaching a firm conclusion, these results support the notion that at least some rDNA types in *Acropora* may be pseudogenes.

There are at least two possible explanations for the origin of different rDNA types within individual *Acropora* colonies and species. First, a hybridization event may have brought together diverged rDNA families and, as the mismatch repair machinery seems sensitive to remote mismatches, high divergence in the spacer regions may have suppressed recombination across the entire rDNA array, impeding concerted evolution (Petit et al. 1991; Muir et al. 2001). In contrast, when parental species have very similar sequence composition, such as between *Armeria villosa* and *Armeria colorata* (Plumbaginacea), where ITS2 differs in only six out of 245 sites, homogenization can occur in only two generations (Fuertes Aguilar et al. 1999). I hypothesize that the rDNA type of clade IVC may be the product of a hybridization event between *A. aspera* and some of the species in clade IVB. Asexual reproduction may also limit the effectiveness of concerted evolution to homogenize divergent rDNA copies, because in asexual taxa gene conversion and crossing over are restricted to mitotic divisions (i.e. in fungi, Pringle et al. 2000). This could be the case in *A. millepora*, which according to population genetic data has one of the highest rates of asexual reproduction in *Acropora* (Ayre and Hughes 2000). Alternatively, hybridization may produce chromosome rearrangements (Rieseberg et al. 1995) that can relocate rDNA copies at different chromosomal positions, reducing the homogenizing action of concerted evolution (Arnheim et al 1980; Muir et al. 2001).

A second possible origin for the divergent rDNA copies is gene duplication, followed by dispersal to nonhomologous, and nonrecombinant, chromosomes (Pringle et al. 2000; Muir et al. 2001). This explanation (and the chromosome rearrangement one) requires an *in situ* hybridization study to determine whether the divergent sequences in *Acropora* are present in more than one nucleolus organizer region (NOR).

Independently of the origin of these divergent rDNA types and although more cDNA sequences are needed before reaching a conclusion, it is likely that nucleolar dominance silenced the rDNA type in clade IVC that then evolved neutrally producing pseudogenes.

```
gemmifera96.2 -*---------T---*****---C----------TTA-C------A---C--T---T---CC-----A--A
gemmifera93.5 -------GC-TGCTT-**---------------CC-A*C------A-A-C--T---T-C----CA--TC--
pulchra80.1   -*---------T---*****---C----------TTA-C------A---C--T---T---C------A---
Aht.1         -------------------------------*A-C-C*-*-C-C-----T--C-------------CG---
hyacinthus32.2-------------------------------*C-C-C*-**C-C--C--T--C-------------AC-A
spicifera78.4 ---------GCG-TT-CC*---------------TCA*-****CT----A--*--G----T------GT-A
hyacinthus1.3 -------------------------------*A-C-C*-**CACT----A**C--CA-TAT-AT---ACGA
cytherea20.3  -------------------------------*A-C-C*-**CCC-----T--C-------------CT---
tenuis18.1    ------C----*---******--C--------C-C-**-**C-C*--CGAC*C--*--C-T--T-T-TT*-
pulchra83.1   --ATCT--CAC*-------------------T-ATCT-*-----C-ATCG-*CT---------TACAT**-
pulchra81.1   -*---------T---*****---C----------TTA-C------A---C--T---T----------AT-A
prolifera22.4 --ATCG--GGATCCA-CCA-CC--CCCAC--GAAAGG-AG--CATCATCGTATATTGACGT-ATATCGTAT
aspera93.3    --AATG--CGATCTT-AC-------------T-ATCT-*------C-C-GC-TA***-C---C-AT-***-
millepora75.3 ----------C---------------------CC-A*C------A-T-C--T--------------T---
aspera85.4    --AACG--CGATCTT-A--------------T-ATCT-*--------C-GC-TA**T--**-*****ACGG
Acy.3         ----------------*--------------*A-C-C*-**C-C-----T--C----G--------CT---
pulchra22.2   -*---------*---****---------------TTA-C------A---C--T---T----------A---
millepora19   --------------T----------------*A-C-C-C-***C-----T--C----G--------CT---
aspera53.4    -------A---*--A-CG*ATCT-TA-CG*-GAATGGNAG---ATC-TCTT-GATT-AGCT----TAACGG
millepora38.8 -C----**-***---*------*-----------TTG-A-A-C--AC***C*CA-C*-----G--*-T--A
hyacinthus16.5-------------------------------*A-C-C*-**C-C--C--T--C----C---------AC-A
Alo.4         ACGCCC----C*--AC-------C----------CTC*-------CA--GTCGT-CG-TGTCC-*T-***G
Ava.2         ACG-------C----**----------------TTA-C------A---C--T---T----------T---
              ITS1.....................................................................
consensus     CG****AT*CGC***TGTGTGAATGG*TAGTCT*YAMTTCTTTGATTGAYATYGAACTATA*TATATWAT*

gemmifera96.2 -*--CA---G--C--T-------**-----------G---------------------------------
gemmifera93.5 -C--CA---G-GA*****-******-----------G---------------------------------
pulchra80.1   ----CA---G--C--T-------**-----------G---------------------------------
Aht.1         ------*-C-*--C**-------------------------------------------------------
hyacinthus32.2T-----*-C-*--C**-------------------------------------------------------
spicifera78.4 T---C-*-G-*---A------------C---------------------------------------
hyacinthus1.3 T-----*-C-*--C-------------------------------------------------------
cytherea20.3  ------*-C-*--C**-----------------C---------------------------------
tenuis18.1    -GAGTG--G-*-AGA-ACGCCATCG----------C-G-------------------------------
pulchra83.1   -****G--G-**A-T-ACGTCATCG---C-------------------------------------
pulchra81.1   TC--CA---G--C--------****-----------G---------------------------------
prolifera22.4 -CACCG----*CCTC-TCGTC-TCG---C--------G-----------A--------C----------
aspera93.3    --C--G--G--GAGA-AGGTCATCG---C-------------------------------------
millepora75.3 -C--CA---G---*---------**-----------G---------------------------------
aspera85.4    AGAC--*GAG-GAGA-ACGTCATCG---C-------------------------------------
Acy.3         ------*CA-**-C----------------------------------------------------
pulchra22.2   -C--CA---G--C--T-----****-----------G---------------------------------
millepora19   ------*-CG-*-*----------------------------------------------------
aspera53.4    AGAC--*GAG-GAGA--CGCCATCG---C-------------------------------------
millepora38.8 -C---AAC-A--C----------**-----------G---------------------------------
hyacinthus16.5T-----*-C-*--C**-------------------------------------------------------
Alo.4         T---*-*-C-**-***-C-*CATCG----------C-G-------------------------------
Ava.2         -C--CA---G--C--T-------------------G---------------------------------
              .....................ITS1/5.8S.....................................
consensus     *AGAA*GA**AA*AGAGA*GAGAGACTCTGCACGGTGAATCTCTCGGCTCGCGCATCGATGAAGAACGCAG
```

Figure 2.1 Alignment of sequence representatives of the *Acropora* ITS1-5.8S-ITS2 region. Dashes represent identity with the consensus sequence and '*' indicate gaps.

```
gemmifera96.2 ------------------------------------------------------------------
gemmifera93.5 -------------------------G----------------------------------------
pulchra80.1   ------------------------------------------------------------------
Aht.1         ------------------------------------------------------------------
hyacinthus32.2-----------------------------------------------------------------
spicifera78.4 -------T-------------------G--T-------C-----------------------C------
hyacinthus1.3 ------------------------------------------------------------------
cytherea20.3  ------------------------------------------------------------------
tenuis18.1    ------------------------------A-----C---C---------------------TCC-A--G-
pulchra83.1   ------------A-------------------------C-----------------------C------
pulchra81.1   ------------------------------------------------------------------
prolifera22.4 ------------------------------------C-----------------------C------
aspera93.3    ------------A-------------------------C-----------------------C------
millepora75.3 ------------------------------------------------------------C------
aspera85.4    ------------A-------------------------C-----------------------C------
Acy.3         ------------------------------------------------------------------
pulchra22.2   ------------------------------------------------------------------
millepora19   ------------------------------------------------------------------
aspera53.4    ------------A-------------------------C-----------------------C------
millepora38.8 T----------------------Y----------------------------------------
hyacinthus16.5-----------------------------T----------------------------------C-------
Alo.4         ----AC----------------------A-----C---C----------------------C*-A--G-
Ava.2         ------------------------------------------------------------------
              ..................................................................
consensus     CCAACTGCGACAGACGTAGAGATCCGATCGTCGATTCTTTGAACGCAAATGGCGCTCGTCTC*TTG*CGAG

gemmifera96.2 ---------------------------*--------------------------------------
gemmifera93.5 -------------------------C**G--C--------------------------------------
pulchra80.1   ---------------------------*--------------------------------------
Aht.1         ------------------------------------------------------------------
hyacinthus32.2-----------------------------------------------------------------
spicifera78.4 ----------G----A--------G--*--------------------------------------
hyacinthus1.3 ------------------------------------------------------------------
cytherea20.3  ----------------------G------------------------------------------
tenuis18.1    A-----G----C--------------C-CG-GG--------CTCT---GCGTGCGTCGCGG----------
pulchra83.1   ------------------------*A-ATT-CC-------*-------T-TCTGTCTGTCTGTCTTT-AGA
pulchra81.1   ---------------------------*--------------------------------------
prolifera22.4 ------------------------A-CCC-CACGAGGC-----------CTT---------------AG
aspera93.3    ------------------------*A-ATT-CC-------*---------TCTGTCTGTGTTT------GA
millepora75.3 --A--------------------*A--A-------------------------------------
aspera85.4    ------------------------*A-ATT-CC-------*---------TCTGTCTGTCTGTCCTT-AGA
Acy.3         ------------------------------------C-------------------------------
pulchra22.2   ---------------------------*--------------------------------------
millepora19   ------------------------------------------------G-------------------
aspera53.4    ------------------------*A-ATT-CC-------*---------TCCATCCATCCATCTTTTGTG
millepora38.8 -------------------------C*-T-----------------GGTTATTCGGG----------
hyacinthus16.5-----------------------------------------------------------------
Alo.4         A----------C-----------G-C-CTC--------C--------GCGTGCGTCGCGT----------
Ava.2         -------------------------C*-T-----------------GGTTATTCGGGT----------
              ..........................5.8S..ITS2.................................
consensus     GCGAGCAAGGCTGTCCGAGCGTCCCTTTGCT*******TTT****ACCCA********************
```

Figure 2.1 continued.

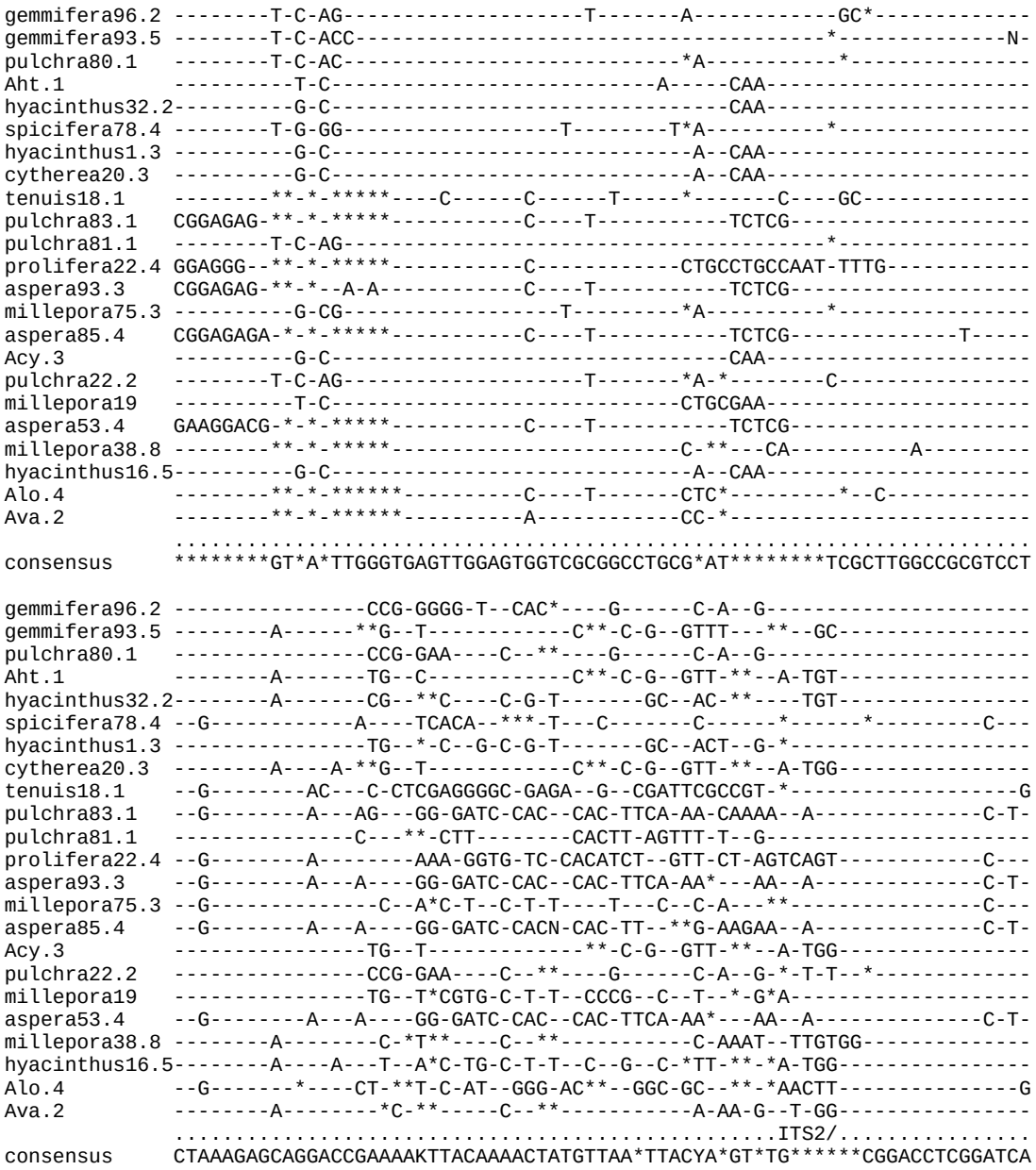

Figure 2.1 continued.

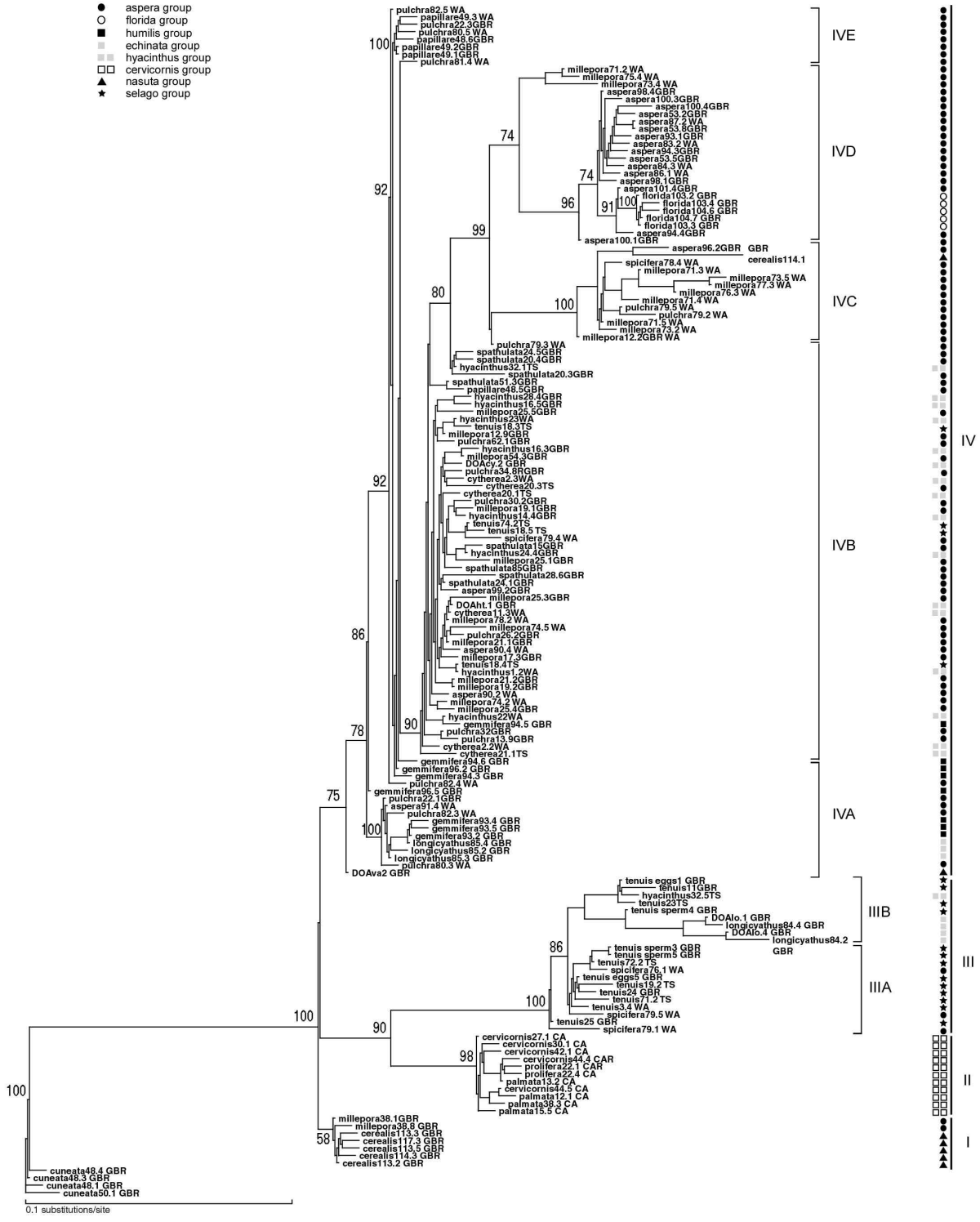
aspera group
florida group
humilis group
echinata group
hyacinthus group
cervicornis group
nasuta group
selago group
IVE
IVD
IVC
IVB
IVA
IIIB
IIIA
IV
III
II
I
0.1 substitutions/site

Figure 2.2 Rooted maximum-likelihood tree of the 5.8S in *Acropora*. Bootstrap values are on branches (1,000 replicates). Major clades and subclades are indicated. Sample codes are as in Table 2.1 in this manuscript, and in Odorico and Miller (1997), van Oppen et al (2000), and van Oppen et al. submitted. A dot and a number after the sample code indicate the clone that was sequenced. Sample names for the *A. hyacinthus* and *A. aspera* groups indicate geographic origin: GBR, Central Great Barrier Reef; TS, Torres Strait; and WA, Western Australia.

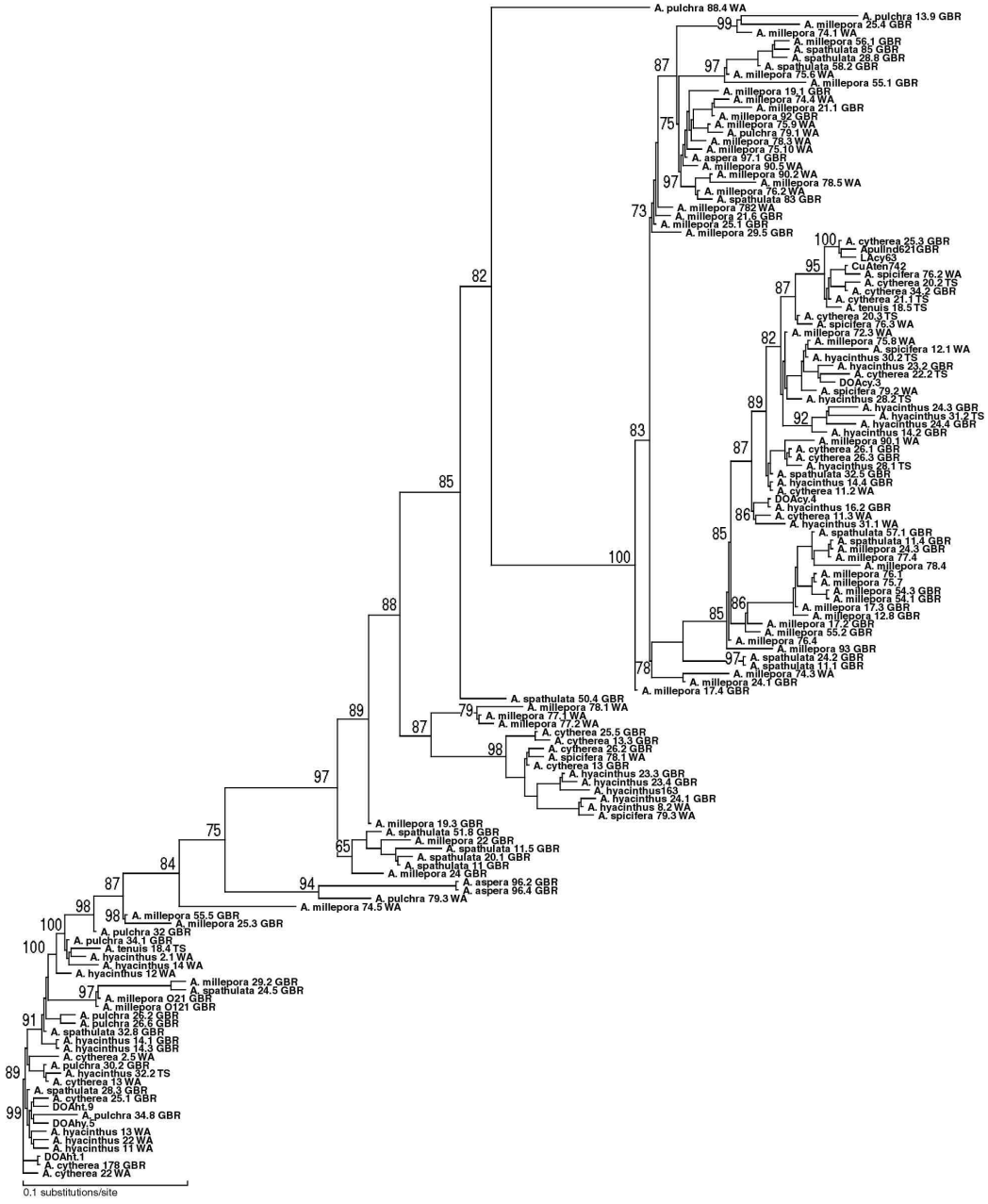

Figure 2.3 Rooted maximum-likelihood tree of the ITS2 region of the *Acropora* samples from sub-clade IVB in the 5.8S phylogeny, using the HKY85 model of sequence evolution. Sample codes are as in Table 2.1 in this manuscript, and in Odorico and Miller (1997), van Oppen et al (2000), and van Oppen et al. submitted.

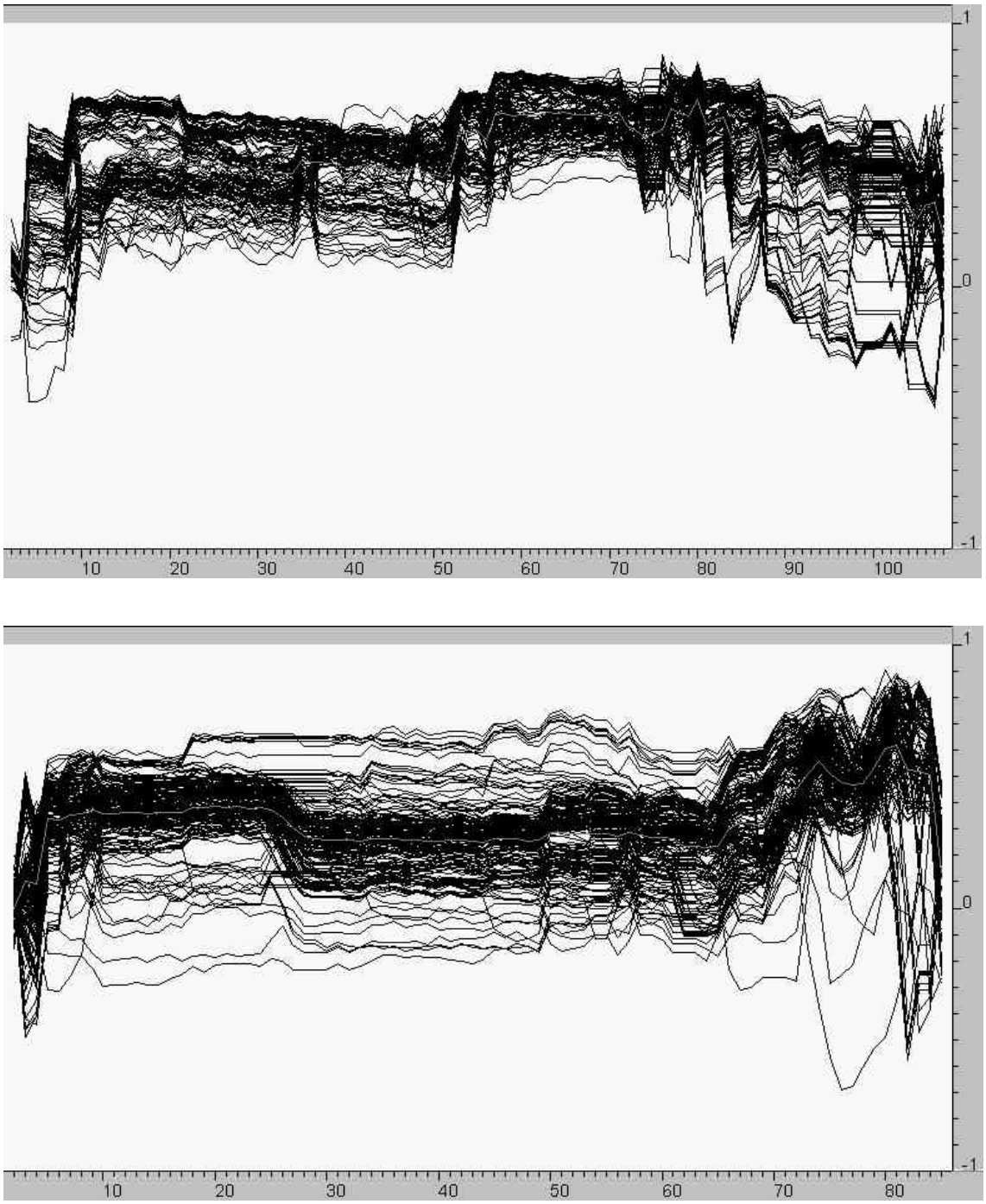

Figure 2.4 Phylogenetic profile of: A) the 5.8S gene in 160 *Acropora* sequences used for the ML phylogeny using a sliding window of 5 differences; and B) the ITS2 region in the 185 *Acropora* sequences used in the ML phylogeny. The most recombinant sequences are listed in Table 2.5. Each line represents a sequence. X axis represents the nucleotide positions along the sequence, while the Y axis corresponds to the correlation coeficient (see text).

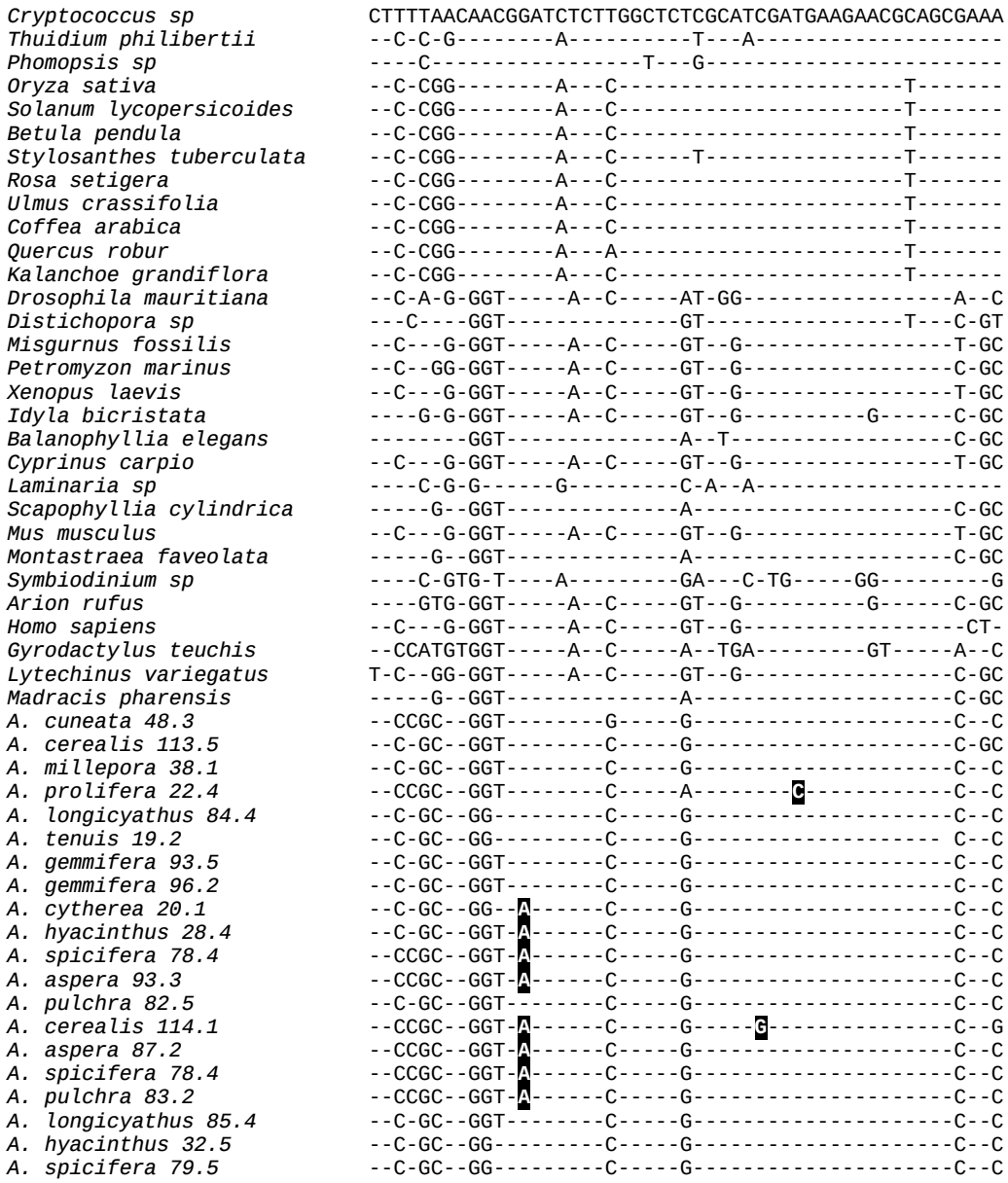

Figure 2.5 Alignment of 5.8S from a broad range of taxa and representative *Acropora* sequences to evaluate mutations at conserved sites. Dashes indicate conserved positions in relation to the sequence on top and '*' indicate gaps. *Acropora* bases in black boxes correspond to mutations in positions that are conserved among the 30 other taxa.

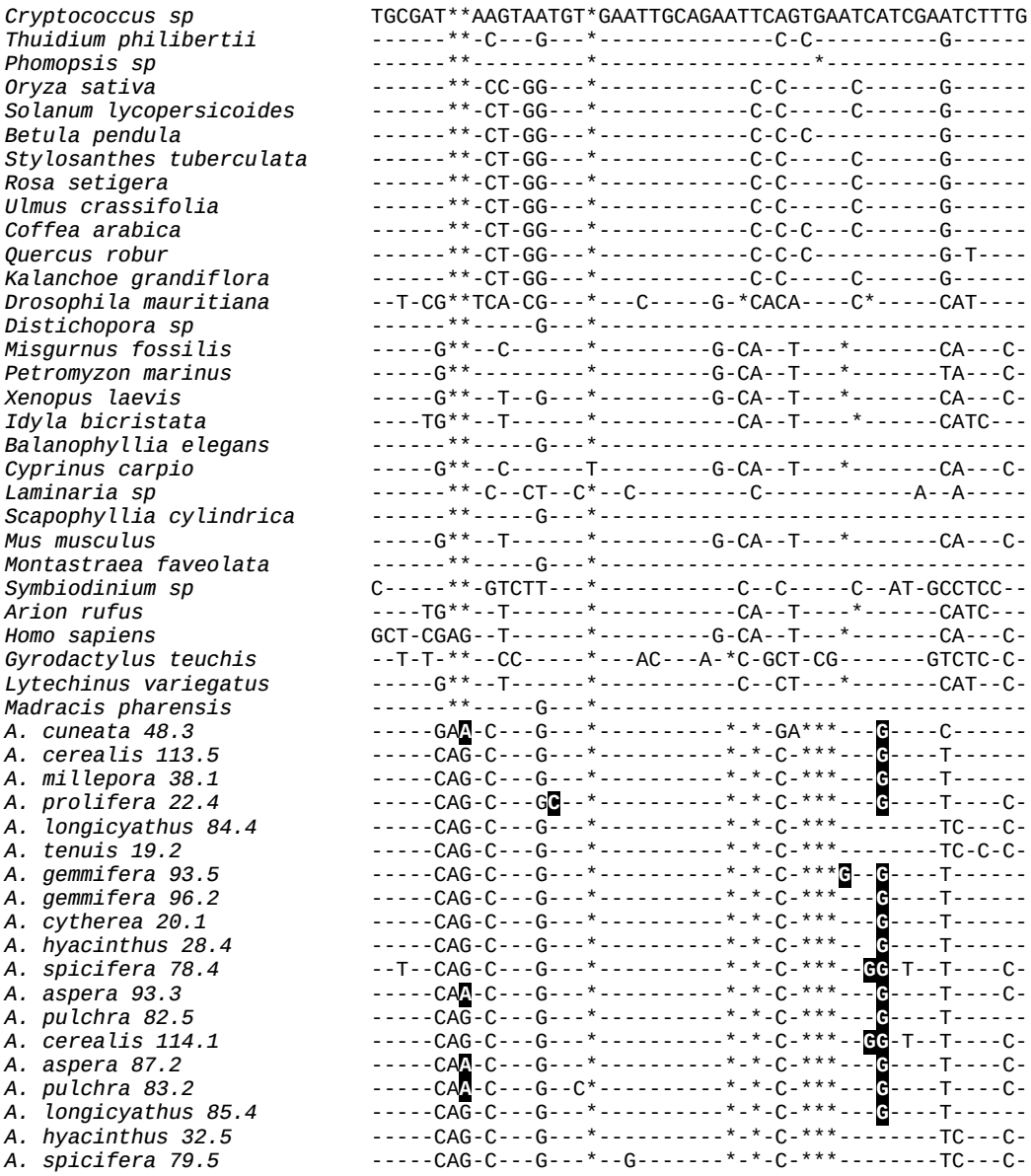

Fig 2.5 continued.

```
Cryptococcus sp            AACGCACCTTGCGCCCGTTGGTAT**TC*CGACGGGCA*TGCCTGTTTGAGTGTCGTG
Thuidium philibertii       ------AG---------AG-C-TG**--**C-A-----*-TT-C-C-A---C---ACC
Phomopsis sp               -------A-------GC-CT-GTA**-T*-CGGA--GCA--------C---C---A-T
Oryza sativa               ------AG---------AG-CC--**C-GGCCGA--GCAC-----CC--G-C---AC-
Solanum lycopersicoides    ------AG---------AA-CC--**-AGGCCGA--GCAC-T---CC--G-C---AC-
Betula pendula             ------AG---------AA-CC-C**CTGGCCGA--GCAC-T---CC--G-----AC-
Stylosanthes tuberculata   ------AG---------AA-CCC-*TAGG-TGA-----*C-----CC--G-----ACC
Rosa setigera              ------AG---------AA-CC--**-AGGCCGA----*C-T---CC--G-C---ACA
Ulmus crassifolia          ------AG---------AA-CCT-**CGGGCCGA--GCAC-T---CC--G-C---ACA
Coffea arabica             ------AG---------AA-CCT-**-AGGCCGA--GCAC-T---CC--G-C---AC-
Quercus robur              ------AG---------AA-CC--**--GGCCGA--GCAC-T---CC--G-----AC-
Kalanchoe grandiflora      ------AG---------AA-CC--**-AGGCGG---GCAA---C-CC--G-C---AC-
Drosophila mauritiana      ------*TA-CGCAGTCCAT-CTG**-T*ATGTACTTT*AATTAA---T-TA--GC--
Distichopora sp            ------AA------T-*-CT-G--**ATC-AGG-A---*------------------C
Misgurnus fossilis         -------A--C--G--CCG---TC**CTC-CGG--C--*C---CCCCC---G----CT
Petromyzon marinus         -------A-----G--CCG--AG-TC-TT-CCG----CAC------C----G----CC
Xenopus laevis             ------------G--CCG---TC**CT*-CCG----CAC------C----G----CT
Idyla bicristata           ------TA-G---G--TCG---CC**AT*-CCGA---CAC---C--C----G----GC
Balanophyllia elegans      ------AA-G----T-T-G---TC**--*-C-G-A---**-T----C--TC--AGTGT
Cyprinus carpio            -------T-----G--CCG---TC**CT*-CCG----CAC------C----G----CT
Laminaria sp               ------T-------TTCCG--ATA**CT*-CTG--AGCA---T---CG-------TGT
Scapophyllia cylindrica    ------AA-G----T-T-G---TC**--**CCA--AGCA--T----C--------AGA
Mus musculus               -------T-GCG----C*G---TC**CT*-CCG----TAC------C----C----GT
Montastraea faveolata      ------AA-G----T-T-G---TC**--*-C-G-A---*--T----C---------GA
Symbiodinium sp            ----T--G----A-T-TCG--AT-**--*-TGA-A-T-*--T---C--C----CTTA-
Arion rufus                ------TA-G---G--TCG---CC**AT*-CCG----CAC---C--C----G----GC
Homo sapiens               ------*------G--CCG---TC**CT*-CCG----TAC------C----C----CT
Gyrodactylus teuchis       ------AA-G---G-TAAG--CT-**G-**TCTTA--CAC-TTC-A-C--------GC
Lytechinus variegatus      -------A-G---G--CGG-CCT-*CG-GGCC--A--CAC---C--CC---G----GT
Madracis pharensis         ------AA-G----T-T-G---TC**--*-C-G-A---*--T----C---------GA
A. cuneata 48.3            ------AA-G----T---C-C-TCG*AG*--G--A---TG---G--C*---A---CCT
A. cerealis 113.5          ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C---CCT
A. millepora 38.1          ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C---CCT
A. prolifera 22.4          ------AA-G----T---CTC-CG**CG*A-G--A---*A-G----CC---C---CAT
A. longicyathus 84.4       ------AA-G----T--CCTC-C-G*AG*G-GAC-AGCAA-G-C--CC---C---CGT
A. tenuis 19.2             ------AA-G----T---CTC-CCGACG*G----A--G*A-G-C--CC---C---CCT
A. gemmifera 93.5          ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C---CCT
A. gemmifera 96.2          ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C---CCT
A. cytherea 20.1           ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C--GCCT
A. hyacinthus 28.4         ------AA-G----T---CTC-TG**CG*A-G--A---*A-G-G--CC---C---CCT
A. spicifera 78.4          ------AA-G----T---CTC-C**GCG*A-G--A---*A-GG---CA---C---CGT
A. aspera 93.3             ------AA-G----T---CTC-CG**CG*A-G--A---*A-G----CC---C---CAT
A. pulchra 82.5            ------AA-G----T---CTC-TG**CG*A-G--A---*A-G----CC---C---CCT
A. cerealis 114.1          ------AA-G----T--**CC-C-CACG*A-G--A---*A-GG---CG---C---CGT
A. aspera 87.2             ------AA-G--------CTC-CG**CG*A-G--A---*A-G----CC---C---CAT
A. pulchra 83.2            ------AA-G----T---CTC-C**GCG*A-G--A---*A-G----CC---C---CAT
A. longicyathus 85.4       ------AA-G----T--**TC-C-TGCG*A-G--A---*A-G----CC---C---CCT
A. hyacinthus 32.5         ------AA-G----T---CTC-T-GACG*G----A--G*A-G-C--GC---C---CCT
A. spicifera 79.5          ------AA-G----T---CTC-CCAACG*G----A--G*A-G-C--CC---C---CCT
```

Fig 2.5 continued.

```
C G C G A[C U C U U A G C G G]U G G A[U C A C]U C - - -[G G C  Xenopus laevis
- - C G A[C U C U U A G C G G]U G G A[U C A C]U C - - -[G G C  Homo sapiens
- - C G A[C U C U U A G C G G]U G G A[U C A C]U C - - -[G G C  Mus musculus
- - C G A[C U C U U A G C G G]U G G A[U C A C]U C - - -[G G C  Rattus norvegicus
- - - G A[C U C U C A A C G G]U G G A[U C A C]U C - - -[G G C  Herdmania momus
G A C G A[C U C U U A A C G G]U G G A[U C A C]U C - - -[G G C  Styela plicata
G A G A U[C U C U U A G C G G]U G G A[U C A C]U C - - -[G G C  Thalia democratica
- - - A A[C U C U A A G C G G]U G G A[U C A C]U C - - -[G G C  Drosophila melanogaster
- - C U A[G C U U C A G C G A]U G G A[U{C}G G U]U - - -[G C A  Caenorhabditis elegans
- - - A A[C C C U A G A C A G]G G G A[U C A C]U U - - -[G G C  Chironomus tentans
- - - o A[C C C U A G G C A G]G G G A[U C A C]U C - - -[G G C  Aedes albopictus
- - - A A[C C C U A G G C A G]G G G A[U C A C]U C - - -[G G C  Anopheles albimanus
- - - - -[C U C U G C A C G G]C G G A[U C U C]U C - - -[G G C  Acropora tenuis 19.2
- - - - -[C U C U G C A C G G]C G G A[U C U C]U C - - -[G G C  A. longicyathus 84.4
- - - - -[C U C C G C A C G G]U G G A[U C U C]U G - - -[G G C  A. cuneata 48.3
- - - - -[C U C C G C A C G G]U G G A[U C U C]U C - - -[G G C  A. prolifera 22.4
- - - - -[C U C U G C A C G G]U G G A[U C U C]U C - - -[G G C  A. millepora 38.1
- - - - -[C U C U G C A C G G]U G G A[U C U C]U C - - -[G G C  A. cerealis 113.2
- - - - -[C U C U G C A C G G]U G G A[U C U C]U C - - -[G G C  A. gemmifera 96.2
- - - - -[C U C U G C A C G G]U G G A[U C U C]U C - - -[G G C  A. gemmifera 93.5
- - - - -[C U C U G C A C G G]U G A A[U C U C]U C - - -[G G C  A. hyacinthus 28.4
- - - - -[C U C C G C A C G G]U G A A[U C U C]U C - - -[G G C  A. aspera 98.4
- - - - -[C U C U G C A C G G]U G G A[U C U C]U C - - -[G G C  A. pulchra 82.5
- - - - -[C U C C G C A C G G]U G A A[U C U C]U C - - -[G G C  A. spicifera 78.4
- - - - -[C U C C G C A C G G]U G A A[U C U C]U C - - -[G G C  A. millepora 77.3
- - - - -[C U C C G C A C G G]U G A A[U C U C]U C - - -[G G C  A. cerealis 114.1
- - - - -[C U C U G C A C G G]U G A A[U C U C]U C - - -[G G C  A. hyacinthus (Aht.1)
- - - - - - - - - -B1 - - - - - - - - - -B2 - - - - - - - - -  Helix numbering euk
```

Figure 2.6 Alignment of 5.8S gene of a wide range of metazoans and of representative sequences from each clade in the ML phylogeny with indications of secondary structure. [and] indicate beginning and end of one strand of a helix; ^ symbolizes][, a new helix starting immediately after the previous one; { and } indicate beginning and end of an internal loop or bulge loop interrupting a helix strand; ▉ highlight a base forming part of a non-standard pair (any pair other than G.C, A.U, or G.U). *A. tenuis* 19.2 - Clade IIIA, *A. longicyathus* 84.4 - Clade IIIB, *A. cuneata* 48.3 - Outgroup, *A. prolifera* 22.4 - Clade II, *A. millepora* 38.1 - Clade I, *A. cerealis* 113.2 - Clade I, *A. gemmifera* 96.2 - Clade IVA, *A. gemmifera* 93.5 - Clade IVA, *A. hyacinthus* 28.4 - Clade IVB, *A. aspera* 98.4 - Clade IVD, *A. pulchra* 82.5 - Clade IVE, *A. spicifera* 78.4 - Clade IVC, *A. millepora* 77.3 - Clade IVC, *A. cerealis* 114.1 - Clade IVC, *A. hyacinthus* Aht.1 - Clade IVB.

```
U C G U G C]- G U C G - A U G A A[G - A A^C G C A]G C - - U A G C -[U  X. laevis
U C G U G C]- G U C G - A U G A A[G - A A^C G C A]G C - - U A G C -[U  H. sapiens
U C G U G C]- G U C G - A U G A A[G - A A^C G C A]G C - - U A G C -[U  M. musculus
U C G U G C]- G U C G - A U G A A[G - A A^C G C A]G C - - U A G C -[U  R. norvegicus
U C G C G A]- G U C G - A U G A A[G - G A^C G C A]G C - - U A A G -[U  H. momus
U C G C G A]- G U C G - A U G A A[G - G]A[C G C A]G C - - C A A G -[U  S. plicata
U C G A G C]- G U C G - A U G A A[G - G]A[C G G A]G C - - U A G C -[U  T. democratica
U C A U G G]- G U C G - A U G A A[G - A A^C G C A]G C - - A A A C -[U  D. melanogaster
U C G A G U]- A U C G - A U G A A[G - A A^C G C A]G C - - U U G C -[U  C. elegans
U C A U G G]- G U C G - A U G A A[G - A C^C G C A]G C - - A A A C -[U  C. tentans
U C G U G G]- A U C G - A U G A A[G - A C^C G C A]G C - - U A A A -[U  A. albopictus
U C A U G G]- A U C G - A U G A A[G - A C^C G C A]G C - - U A A A -[U  A. albimanus
U C G C G C]- A U C G - A U G A A[G - A]A[C G C A]G U - - C A A C -[U  A. tenuis 19.2
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. longicyathus 84.4
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. cuneata 48.3
U C A C G C]- A U C G - A C G A A[G - A A^C G C A]G C - - C A A C -[U  A. prolifera 22.4
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. millepora 38.1
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A G C -[U  A. cerealis 113.2
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. gemmifera 96.2
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. gemmifera 93.5
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. hyacinthus 28.4
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. aspera 98.4
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. pulchra 82.5
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. spicifera 78.4
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. millepora 77.3
U C G C G C]- A U G G - A U G A A[G - A A^C G C A]G C - - C A A G -[U  A. cerealis 114.1
U C G C G C]- A U C G - A U G A A[G - A A^C G C A]G C - - C A A C -[U  A. hyacinthus (Aht.1)
- -B3 - - - - - - - - - - - - - - - -B4 - - - -B5 - - - - - - - - - -  Helix numbering euk

G - C G]- - A G A A U U A[G U - G{U G - A A}U U]G - - C A G - -[G A{- C A  X. laevis
G - C G]- - A G A A U U A[A U - G{U G - A A}U U]G - - C A G - -[G A{- C A  H. sapiens
G - C G]- - A G A A U U A[A U - G{U G - A A}U U]G - - C A G - -[G A{- C A  M. musculus
G - C G]- - A G A A U U A[A U - G{U G - A A}U U]G - - C A G - -[G A{- C A  R. norvegicus
G - C G]- - A G A A G U G[G U - G{U G - A A}U U]G - - C A G - -[A A{- C A  H. momus
G - C G]- - A G A A G U A[A U - G{U G - A A}U]U G - - C A G A - -[A{- C A  S. plicata
C - C G]- - A G A A G U A[A U - G{U G - A A}U]U G - - C A G G - -[A{- C A  T. democratica
G - U G]- - C G U C A U C[G U - G{U G - A A C}U]G - - C A G - -[G{A - C A  D. melanogaster
G - C G]- - U U A C U U A[C C - A{C G - A A}U]U G - - C A G - - -[A{- C G  C. elegans
G - C G]- - C G U C G C C[A U - G{U G - A A C}U]G - - C A G - -[G{A - C A  C. tentans
G - C G]- - C G U C A G A A[U - G{U G - A A C}U]G - - C A G - -[G{A - C A  A. albopictus
G - C G]- - C G U C A G A A[U - G{U G - A A C}U]G - - C A G - -[G{A - C A  A. albimanus
G - C G]A C A G A C G U A[G U - G{U G - A A}U]U G - - C A G - -[A{U - C C  A. tenuis 19.2
G - C G]A C A G A C G U A[G U - G{U G - A A}U]U G - - C A G - -[A{U - C C  A. longicyathus 84.4
G - C G]A G A A A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. cuneata 48.3
G - C G]A C A G A C G U A[G C - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. prolifera 22.4
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. millepora 38.1
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. cerealis 113.2
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. gemmifera 96.2
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. gemmifera 93.5
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. hyacinthus 28.4
G - C G]A C A A A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. aspera 98.4
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. pulchra 82.5
G - U G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. spicifera 78.4
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. millepora 77.3
G - C G]A C A G A C G U A[G U - G{U G - A A]U]U G - - C A G - -[A{U - C C  A. cerealis 114.1
G - C G]A C A G A C G U A[G U -  {U G - A A]U]U G - - C A G - -[A{U - C C  A. hyacinthus (Aht.1)
- - B5'-  - - - - - - - - - -B6 - - - - - - - - - - - - - - - - - - - - -  Helix numbering euk
```

```
C A U U G A U}C A U]C G A C A - - - - - - - - C[U U C]G A A -[C G C]A  Xenopus laevis
C A U U G A U}C A U]C G A C A - - - - - - - - C[U U C]G A A -[C G C]A  Homo sapiens
C A U U G A U}C A U]C G A C A - - - - - - - - C[U U C]G A A -[C G C]A  Mus musculus
C A U U G A U}C A U]C G A C A - - - - - - - - C[U U C]G A A -[C G C]A  Rattus norvegicus
C A U U G A A}C G U]C G A C - - - - - - - - - C[U U C]G A A -[C G C]G  Herdmania momus
C A U U G A A}C G U]C G A C C - - - - - - - - U U[C U]G A A - C[G C]G  Styela plicata
C A U U G A G}C A U]C G A U A - - - - - - - - U U[C U]G A A - C[G C]G  Thalia democratica
C A U - G A A}C A U]C G A C A - - - - - - - - U[U U U]G A A - C[G C]A  Drosophila melanogaster
C U U A G A G}U G G]U G A A A - - - - - - - - U[U U C]G A A - C[G C]A  Caenorhabditis elegans
C A U - G A U}C A U]U G A C A - - - - - - - - U[G U U]G A A - C[G C]A  Chironomus tentans
C A U - G A A}C A]C C G A C A - - - - - - - - C[G U U]G A A - C[G C]A  Aedes albopictus
C A U - G A A}C A]C C G A C A - - - - - - - - C[G U U]G A A -[C G]- A  Anopheles albimanus
G A U - - - -}C A U]C G A U C - - - - - - - - C C[U C]G A A - C[G C]A  Acropora tenuis 19.2
G A U - - - -}C A U]C G A U C - - - - - - - - C[U U C]G A A - C[G C]A  Acropora longicyathus 84.4
A A U - - - -}C G U]C G A C U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora cuneata 48.3
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U C]G A A - C[G C]A  Acropora prolifera 22.4
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora millepora 38.1
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora cerealis 113.2
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora gemmifera 96.2
G G U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora gemmifera 93.5
G A U - - - -}U G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora hyacinthus 28.4
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U C]G A A - C[G C]A  Acropora aspera 98.4
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora pulchra 82.5
G A U - - - -}G G U]U G A U U - - - - - - - - C[U U C]G A A - C[G C]A  Acropora spicifera 78.4
G A U - - - -}G G U]U G A U U - - - - - - - - C[U U C]G A A - C[G C]A  Acropora millepora 77.3
G A U - - - -}G G U]U G A U U - - - - - - - - C[U U C]G A A - C[G C]A  Acropora cerealis 114.1
G A U - - - -}C G U]C G A U U - - - - - - - - C[U U U]G A A - C[G C]A  Acropora hyacinthus (Aht.1)
- - - - - -B6'- - - - - - - - - - - - - - - - - -B4'- - - - - - -B7 -  Helix numbering euk

C C U U - - - - - -[G C G^G C C C C G G G]- - - - - - - - - - - U U C C  Xenopus laevis
C U U - - - - - - -[G C G^G C C C C G G G]- - - - - - - - - - - U U C C  Homo sapiens
C U U - - - - - - -[G C G^G C C C C G G G]- - - - - - - - - - - U U C C  Mus musculus
C U U - - - - - - -[G C G^G C C C C G G G]- - - - - - - - - - - U U C C  Rattus norvegicus
A A U G - - - - - -[G C G^G U C U C G G G]- - - - - - - - - - - U U A -  Herdmania momus
A A U U - - - - - -[G C]G[G U C U C G G G]- - - - - - - - - - - U C A A  Styela plicata
U A U U - - - - - -[G C]G[G C C U C G G G]- - - - - - - - - - - U - A A  Thalia democratica
U A U C - - - - - -[G C^A G U C C A U G C]- - - - - - - - - - - U G U -  Drosophila melanogaster
U A - - - - - - - -[G C^A C C A A C U G G]- - - - - - - - - - - G C C U  Caenorhabditis elegans
U A U U - - - - - -[G C^G C C U U A U A C A]U U U G G U U C U C - - - -  Chironomus tentans
U A U U - - - - - -[G C^A C A U C G U A C]- - - - - - - - - - - C U C C  Aedes albopictus
U A U U - - - - - - G[C G^C A U U G C A C]G - - - - - - - - - - A C U C  Anopheles albimanus
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U C C G  Acropora tenuis 19.2
A A U G - - - - - -[G C^G C U C G C C U C U]- - - - - - - - - - - C U G A  Acropora longicyathus 84.4
A A U G - - - - - -[G C^G C U C G U C G C]- - - - - - - - - - - U U C G  Acropora cuneata 48.3
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U C G C  Acropora prolifera 22.4
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora millepora 38.1
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora cerealis 113.2
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora gemmifera 96.2
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora gemmifera 93.5
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora hyacinthus 28.4
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U C G C  Acropora aspera 98.4
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora pulchra 98.4
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U C G C  Acropora spicifera 78.4
A A U G - - - - - -[G C^G C U C A U C U]- - - - - - - - - - - - U C G C  Acropora millepora 77.3
A A U G - - - - - -[G C^G C U C G C C U C]- - - - - - - - - - - U C A C  Acropora cerealis 114.1
A A U G - - - - - -[G C^G C U C G U C U C]- - - - - - - - - - - U U G C  Acropora hyacinthus (Aht.1)
- - - - - - - - - -B7'- - - -B8 - - - - - - - - - - - - - - - - - - -  Helix numbering euk
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- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C C  Xenopus laevis
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C C  Homo sapiens
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C C  Mus musculus
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C C  Rattus norvegicus
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A[U C C  Herdmania momus
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C C  Styela plicata
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - U[C C U  Thalia democratica
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G C U  Drosophila melanogaster
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[C C A  Caenorhabditis elegans
U U U A U A A U A U A C A C A A A A U U U A U A A U G U G G A A C[U G U A  Chironomus tentans
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A[G U A  Aedes albopictus
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A[G U G  Anopheles albimanus
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A C[G G G  Acropora tenuis 19.2
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G G G G  A. longicyathus 84.4
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A[G C G  Acropora cuneata 48.3
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora prolifera 22.4
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora millepora 38.1
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora cerealis 113.2
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora gemmifera 96.2
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora gemmifera 93.5
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora hyacinthus 28.4
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora aspera 98.4
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora pulchra 82.5
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora spicifera 78.4
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A[A G  Acropora millepora 77.3
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora cerealis 114.1
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -[G A G  Acropora hyacinthus Aht.1
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Helix numbering euk

G G G G C]C A[C G C C U G U - - - C U G A G{G}G U C]G C U C C - - - -  Xenopus laevis
G G G G C]U A[C G C C U G U - - - C U G A G{C}G U C]G C U U - - - - -  Homo sapiens
G G G G C]U A[C G C C U G U - - - C U G A G{C}G U C]G G U U - - - - -  Mus musculus
G G G G C]U A[C G C C U G U - - - C U G A G{C}G U C]G C U - - - - - -  Rattus norvegicus
G G G A C]C A[C G U C U G{C - - -}C U C A G{G}G U U]G C G A - - - - -  Herdmania momus
G G G A C]C G[C G U C U G{C - - -}C U C A G{G}G U C]G C - - - - - - -  Styela plicata
G G G G C]C A[C G U C C G{U - - -}C U C A G{G}G U C]U U - - - - - - -  Thalia democratica
U G G A C U]A[C A U A U G G - - - U U G A G{G}G U U G U A U]U A U - -  Drosophila melanogaster
G U U G G U]A[C G U C U G G - - - U U C A G{G}G U U]G U U - - - - - -  Caenorhabditis elegans
U A A G G U]A[C A U A U G G - - - U U G A G{U}G U C G U A A]A U U - -  Chironomus tentans
C G A U G U]A[C A C A U U U - - - U U G A G{U}G C C U A U]U A U C A -  Aedes albopictus
C G A U G]U A[C A C A U U U - - - U U G A G{U}G C C C A C]A U U C A C  Anopheles albimanus
A C G A G C]G[A G G C C G U - - - C C G A G{C}G U C C]C U C - - - - -  Acropora tenuis 19.2
A C G A G C]A[A G G C C G U - - - C C G A G{C}G U C C]G U C - - - - -  Acropora longicyathus 84.4
G C G A G C]A[U G G C C G G - - - U C G A G{A}G U C C]C U U - - - - -  Acropora cuneata 48.3
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]A U C - - - - -  Acropora prolifera 22.4
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U C - - - - -  Acropora millepora 38.1
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U C - - - - -  Acropora cerealis 113.2
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U U - - - - -  Acropora gemmifera 96.2
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U C - - - - -  Acropora gemmifera 93.5
G C G A G C]A[A G G C G G U - - - C C G A G{C}G U C C]C U U - - - - -  Acropora hyacinthus 28.4
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]A U A - - - - -  Acropora aspera 98.4
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U U - - - - -  Acropora pulchra 82.5
G C G A G C]A[A G G G U G U - - - C A G A G{C}G U C C]G U U - - - - -  Acropora spicifera 78.4
G C G A G C]A[A G G G U G U - - - C A G A G{U}G U C U]G U U - - - - -  Acropora millepora 77.3
G C G A G C]A[A G G G U G U - - - C G G A G{C}G U C C]G U U - - - - -  Acropora cerealis 114.1
G C G A G C]A[A G G C U G U - - - C C G A G{C}G U C C]C U U - - - - -  Acropora hyacinthus (Aht.1)
-B8'- - - - - - - -B9 - - - - - - - - - - - - - - - - - - - - - - - -  Helix numbering euk
```

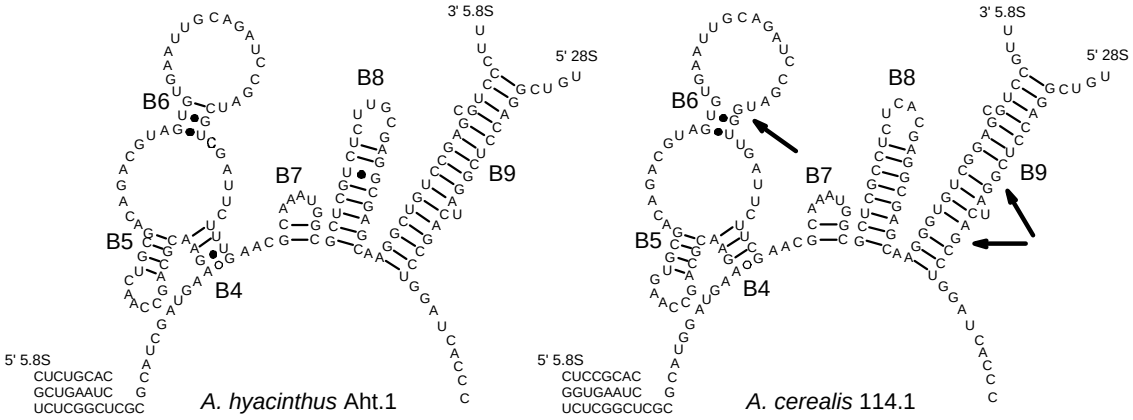


Figure 2.7 Folding of 5.8S rRNA sequences from *A. hyacinthus* (from clade IVB in Figure 2.2) and *A. cerealis* (from clade IVC in Figure 2.2) following Odorico and Miller (1997). Helices numbers after Wuyts (2001). The arrows indicate non-compensatory mutations.

Table 2.1 Samples and rDNA sequence codes. The number after the dot for each sequence indicates the clone sequenced. Location codes used: WA = Western Australia, GBR = Great Barrier Reef, TS = Torres Srait.

Species Group	Species	Collection location	Sequence Codes	Sequenced by
hyacinthus	*Acropora hyacinthus*	Coral Bay, WA	1.1, 1.2, 1.3, 1.4, 1.5, 2.1, 2.2, 2.3, 2.4, 8.2	L. M. Márquez
	A. hyacinthus	Heron Is., GBR	14.1, 14.2, 14.3, 14.4, 14.7	
		Lizard Is., GBR	16.2, 16.3, 16.4, 16.5, 16.9	
		Britomart, GBR	23.2, 23.3, 23.5, 24.1, 24.2, 24.3, 24.4, 28.4	
		Dungeness, TS	28.1, 28.2, 30, 30.2, 31.1, 31.2, 32, 32.1, 32.5	
	A. cytherea	Dampier, WA Bundegi, WA	1, 1.2, 1.5, 2.2, 2.3, 11.2, 11.3, 18.2, 18.5	
		Heron Is., GBR	13, 13.3, 13.5	
		Britomart, GBR	25.3, 25.5, 26.1, 26.2, 26.3	
		Trunk, GBR	34.2	
		Dungeness, TS	20.1, 20.2, 20.3, 21.1, 21.2, 22.2,	
selago	*A. tenuis*	Dungeness, TS	18, 18.1, 19.2	
		Cumberland, TS	71.1, 71.2, 71.5, 72.2,	
		Coral Bay, WA	3.4	
aspera	*A. spicifera*	Coral Bay, WA	9.1, 80.1	
		Bundegi, WA	12.1, 12.4, 75.1, 75.2, 75.4, 76.1, 76.3, 76.4, 78.1, 78.3, 78.4, 79.1, 79.5, 79.3	
	A. aspera	Coral Bay, WA	84.1, 84.2, 84.3, 84.4, 84.5, 85.1, 85.2, 85.3, 85.4, 85.5, 86.1, 86.2, 86.3, 86.4, 87.2, 87.3, 87.4, 87.5	M. J. H van Oppen
	A. millepora	Coral Bay, WA	71.1, 71.2, 71.3, 71.4, 71.5, 72.1, 72.2, 72.3, 72.4, 72.5, 73.2, 73.4, 73.5, 74.1, 74.2, 74.3, 74.4, 74.5	

Table 2.1 continued

Species Group	Species	Collection location	Sequence Codes	Sequenced by
	A. millepora	Bundegi, WA	75.1, 75.2, 75.3, 75.4, 75.5, 75.6, 75.7, 75.8, 75.9, 75.10, 76.1, 76.2, 76.3, 76.4, 76.5, 77.1, 77.2, 77.3, 77.4, 78.1, 78.2, 78.4, 78.5	M. J. H van Oppen
	A. pulchra	Bundegi, WA	79.1, 79.2, 79.3, 79.5, 80.1, 80.2, 80.3, 80.4, 80.5, 81.1, 81.3, 81.4, 81.5, 82.1, 82.2, 82.3, 82.4, 82.5, 83.1, 83.2, 83.3, 83.4, 83.5, 88.1, 88.2, 88.3, 88.4, 89.1, 89.3, 89.5, 90.2, 90.4, 91.1, 91.2, 91.3, 91.4	
nasuta	*A. cerealis*	Trunk,GBR	113.2, 113.3, 113.5, 114.1, 114.2, 114.3, 114.4, 117.3, 117.5	J. B. Mackenzie
echinata	*A. longicyathus*	Trunk,GBR	84.1, 84.2, 84.3, 84.4, 85.2, 85.3, 85.4, 85.5, 86.1	
humilis	*A. gemmifera*	Trunk,GBR	93.2, 93.3, 93.4, 93.5, 94.3, 94.5, 94.6, 96.2, 96.5	
Subgenus *Isopora*	*A. cuneata*	Britomart. GBR	48.1, 48.3, 48.4, 48.5, 50.1, 50.2, 50.4, 50.6	

Table 2.2 Identical *Acropora* 5.8S sequences in the original data set of 416.

Sequence in tree (Clade)	Identical sequences
aspera 98.4 (IVD)	*aspera* 53.4, 84.1, 84.2, 84.4, 84.5, 85.1, 85.2, 85.3, 85.4, 85.5, 86.2, 86.3, 86.4, 87.3, 87.4, 87.5, 92.2, 92.4, 93.3, 93.5, 94.1, 94.2, 94.5, 96.3, 96.5, 98.2, 98.3, 98.5, 99.3, 100.2, 101.3, 103.3, 103.5, 104.7, 104.8, ***florida*** 103.2, ***pulchra*** 30.1, 83.1, 83.3, 83.4, 83.5
aspera 87.2 (IVD)	*aspera* 53.7, 53.8
aspera 101.4 (IVD)	*aspera* 92.1, 92.3
aspera 93.1 (IVD)	*aspera* 93.4
aspera 96.2 (IVC)	*aspera* 96.4
pulchra 82.5 (IVE)	*pulchra* 13.2, 22.2, 26.1, 80.1, 80.2, 80.4, 81.1, 81.5, 82.1, 82.2, 88.1, 88.2, 88.3, 89.1, 89.3, 89.5, 91.1, 91.3, ***spathulata*** 68.2, 68.5, 68.9, ***papillare*** 48.1, 48.2, 49.1, 49.2, 49.4, 60.1, 60.5
pulchra 80.5 (IVE)	*pulchra* 88.4
pulchra 22.1 (IVA)	*pulchra* 13.1, 81.3, 88.5, 89.4, 91.2, 91.4,
pulchra 26.2 (IVB)	*millepora* 90.3
millepora 38.1 (I)	*millepora* 38.10
*papillare*48.6 (IVE)	*aspera* 97.5
millepora 71.2 (IVD)	*millepora* 75.1, 75.2, 75.3, 75.5, 79.4
millepora 73.2 (IVC)	*millepora* 73.1, 73.3
millepora 77.3 (IVC)	*millepora* 72.1, 72.2
millepora 19.2 (IVB)	*millepora* 21.2
hyacinthus DOAht.1 (IVB)	***millepora*** 2, 9.2, 9.3, 9.4, 11, 12.1, 12.8, 17.2, 19, 19.3, 20.1, 21, 21.1, 21.6, 22, 24, 25.1, 25.2, 26.6, 29.2, 29.3, 29.5, 34.1, 50.4, 55.5, 56.1, 56.2, 71.1, 72.3, 72.4, 72.5, 74.1, 74.3, 74.4, 75.6, 75.7, 75.8, 75.9, 75.10, 76.1, 76.2, 76.4, 76.5, 77.1, 77.2, 77.4, 78.1, 78.4, 78.5, 90.5, ***cytherea*** 1, DO3, DO4, DO8, 6.2, 6.3, 11.2, 11.3, 13, 13.3, 13.5, 18.2, 18.5, 20.2, 22.2, 25.3, 25.5, 26.1, 26.2, 26.3, 34.2, ***hyacinthus*** DO2, DO5, DO9, 1.1, 1.3, 1.4, 1.5, 2.1, 2.4, 8.2, 14.1, 14.2, 14.3, 14.7, 16.2, 16.4,16.9, 23.2, 23.3, 23.4, 23.5, 24.1, 24.2, 24.3, 28.1, 28.2, 30, 30.2, 31.1, 31.2, 32, ***spicifera*** 9.1, 12.1, 12.4, 75.1, 75.2, 75.4, 76.3, 76.4, 78.1, 78.3, 79.3, 80.1, ***spathulata*** 1.6, 8.3, 11.1, 11.4, 11.5, 12.9, 16, 17.4, 24.1, 24.2, 24.3, 28.3, 28.8, 32.5, 32.8, 50.1, 51.8, 54.1, 57.1, 57.2, 58.2, ***aspera*** 97.1, 97.3, ***papillare*** 55.1, 55.2, 62.2, 62.3, ***pulchra*** 79.1, ***valida*** 17.3, 17.8
hyacinthus 1.2 (IVB)	*hyacinthus* 18.4
*cytherea*DOAcy.2 (IVB)	*cytherea* 17.2, 17.4
cytherea 2.2 (IVB)	***millepora*** 90.1, ***spicifera*** 79.2
tenuis sperm 3 (IIIA)	*tenuis* sperm 5
tenuis sperm 4 (IIIB)	***cytherea*** WA 1.2, 1.5, ***tenuis*** sperm 1, sperm 2, eggs3, eggs 5, 2.1, 3.1, 18, 18.1, 71.1, 71.5, ***hyacinthus*** 31.5, 32.4
tenuis 72.2 (IIIA)	*tenuis* 1
tenuis 74.2 (IVB)	***cytherea*** 21.2, ***spicifera*** 76.2
*cuneata*48.3(outgroup)	*cuneata* 48.5, 50.2, 50.4, 50.6
*longicyathus*84.4 (IIIB)	*longicyathus* 84.1
*longicyathus*85.3 (IVA)	*longicyathus* 85.5, 86.1
gemmifera 93.2 (IVA)	*gemmifera* 93.3 ***longicyathus*** 85.4
cerealis 113.2 (I)	*cerealis* 114.2, 114.4, 117.5

Table 2.3 Mean base compositions (%) and ranges of Kimura Two-Parameter pairwise sequence distances (%) for the 5.8S and the ITS2 region of *Acropora*. For the base composition, standard deviations are given in parentheses. Maximum distance between *Isopora* and *Acropora* are also given between parentheses.

Region	length	A	C	G	T	Distance range
5.8S	166 bp	20.73	28.83	28.32	22.12	0.00-20.33
		(0.80)	(1.26)	(0.94)	(1.31)	(22.77)
ITS2	200 bp	22.87	23.85	28.00	25.28	0.00-46.64
		(2.73)	(2.70)	(2.16)	(2.44)	(59.48)

Table 2.4 AMOVA between *Acropora* species and *Acropora* clades in the ML phylogeny

Source of variation	d.f.	Sum of squares	Variance components	*P*	Percentage of variation
Species					
Among	14	696.127	4.18531 Va	<0.00001	56.11
Within	161	527.084	3.27381 Vb		43.89
Total	175	1223.210	7.45913		
Clades					
Among	11	626.129	4.07751 Va	<0.00001	74.13
Within	165	234.816	1.42313 Vb		25.87
Total	176	860.944	5.50063		

Table 2.5 *Acropora* 5.8S and ITS2 sequences with the highest recombination in the phylogenetic profiles generated by Phylpro in Figure 2.4.

5.8S	ITS2
A. spicifera 76.1, 79.1, 79.5,	*A. pulchra* 13.9, 22.3, 79.3, 88.4
A. tenuis sperm4, eggs5, 72.2, 19.2, 2.5, 3.4	*A. papillare* 48.6
A. millepora 38.1, 38.3,	*A. spathulata* 11.1, 11.5, 24.2, 24.5, 68.2, 68.9
A. longicyathus DOAlo1, 84.2, 84.4, 85.2, 85.3, 85.4	*A. millepora* 21.1, 25.4, 29.2, 38.1, 38.8, 74.1, 74.5
A. gemmifera 93.5, 96.5	*A. aspera* 88.4, 97.5
A. cerealis 113.2, 113.3, 113.5, 114.1, 114.3, 117.3	*A. spicifera* 12.1
A. palmata 12.1, 13.2, 15.5, 38.3	
A. cervicornis 27.1, 30.1, 44.5	
A. prolifera 22.1, 22.4	
A. cuneata 48.1, 48.4, 48.3, 50.1	

Table 2.6 Relative-rate test for *Acropora* 5.8S clades in ML phylogeny. b_A and b_B are average number of substitutions per site; $\delta = b_A - b_B$. Z-scores are the values from the standardized normal distribution (i.e. 1.96 is the value for $\alpha = 0.05$). * indicates $p<0.05$.

Outgroup	Cluster A	B	b_A[b]	b_B[b]	δ[c]	*z*SCORE
Isopora	II+III+IV	I	0.0478	0.0026	0.0451	4.295*
I	IV	II+III	0.0296	0.0777	0.0480	2.582*
I	III	II	0.0818	0.0488	0.0330	1.135
II+III	IVB-IVE	IVA	0.0263	0.0042	0.0221	1.937
IVA	IVB+IVC+IVD	IVE	0.0362	0.0007	0.0355	2.787*
IVE	IVC+IVD	IVB	0.0384	0.0067	0.0318	2.402*
IVB	IVC	IVC	0.0201	0.0338	-0.0137	0.694

Table 2.7 Absolute number of deamination-like substitutions in 68 methylation and 21 non-methylation sites of the 5.8S in *Acropora* in comparison to the outgroup (*A. cuneata*).

Clade	Methylation sites	Other sites	χ^2 values obtained in comparison with the outgroup (*A. cuneata*)	*P*
I	3	0		
II	3	0	NA	NA
III	5	1	0.563	1.000
IVA	2	0	NA	NA
IVB	9	5	1.518	0.512
IVC	10	4	0.852	0.569
IVD	3	1	0.875	1.000
IVE	2	1	1.200	1.000

Table 2.8 Number of mutations in 45 conserved positions of the 5.8S

Clades (representative sequences used) or Introgressed sequences	Number of mutations
Outgroup (*cuneata* 48.3)	4
Clade I (*cerealis* 113.5 *millepora* 38.1)	1
Clade II (*prolifera* 22.4)	3
Clade III (*longicyathus* 84.4 & *tenuis* 19.2)	2
Clade IVA (*gemmifera* 93.5 *gemmifera* 96.2)	2
Clade IVB (*cytherea* 20.1)	2
Clade IVB (*hyacinthus* 28.4)	3
Clade IVC (*spicifera* 78.4)	3
Clade IVD (*aspera* 93.3)	3
Clade IVE (*pulchra* 82.5)	1
cerealis 114.1	5
pulchra 83.2	3
longicyathus 85.4	1
hyacinthus 32.5	1
spicifera 79.5	1

Chapter 3 Sympatric populations of the highly cross-fertile coral species *Acropora hyacinthus* and *A. cytherea* are genetically distinct

Abstract

High cross-fertilization rates *in vitro* and non-monophyletic patterns in molecular phylogenies challenge the taxonomic status of species in the coral genus *Acropora*. Here I present data from eight polymorphic allozyme loci that indicate small but significant differentiation between sympatric populations of *A. cytherea* and *A. hyacinthus* (F_{ST}= 0.025 to 0.068, p<0.05), a pair of acroporid corals with very high interspecific fertilization rates *in vitro*. Although no fixed allelic differences were found between these species, the absence of genetic differentiation between widely allopatric populations suggests that allele frequency differences between *A. cytherea* and *A. hyacinthus* in sympatry are biologically significant. In contrast, populations of *A. tenuis*, a species which spawns 2-3 hours earlier and shows low cross-fertilization rates with congeners *in vitro,* were clearly distinct from *A. cytherea* and *A. hyacinthus* (F_{ST}=0.427 to 0.465, p<0.05). Moreover, allopatric populations of *A. tenuis* differed significantly, possibly as a consequence of its relatively short period of larval competency. These results effectively rule out the possibility that *A. hyacinthus* and *A. cytherea* are morphotypes within a single species, and suggest that hybridization occurs relatively infrequently between these taxa in nature.

Introduction

Although molecular phylogenetic analyses suggest that interspecific hybridization has played a major role in coral evolution (Odorico and Miller 1997; Hatta et al. 1999; Fukami et al. 2000; Diekmann et al. 2001; van Oppen et al. 2001a), alternative hypotheses to explain observed patterns of allele and haplotype sharing cannot be rigorously excluded. Two alternative hypotheses that have been widely discussed are 1) that cross-fertile coral species are morphotypes within phenotypically plastic species (Willis et al. 1997), or 2) that these patterns of allele and haplotype sharing reflect recent speciation and retention of ancestral polymorphisms (Diekmann et al. 2001; van Oppen et al. 2001a). Population genetic approaches may have greater potential to distinguish between the occasional hybridization versus the “morphologically plastic species” hypothesis than phylogenetic analyses, because comparisons are based on differences in allele frequencies, which can occur over much shorter time frames than the mutational changes on which phylogenetic analyses are based. Thus, sympatric populations of two species that hybridize frequently would be expected to be more similar than allopatric populations within each species. Conversely, no or infrequent hybridization is predicted to lead to greater allele frequency differences between species in sympatry than between allopatric populations within species.

To better understand relationships between cross-fertile *Acropora* species, I compared genetic differentiation between sympatric and allopatric (central GBR versus West Australian) populations of *A. cytherea*, *A. hyacinthus* and *A. tenuis* using allele frequencies at eight polymorphic allozyme loci. *A. cytherea* and *A. hyacinthus* have one of the highest rates of interspecific fertilization *in vitro* (mean = 50%) (Willis et al. 1997), whereas *A. tenuis* spawns 2-3 hours earlier than its congeners during mass spawning events and also has low interspecific compatibility in breeding trials. The comparison of sympatric vs. allopatric populations of species with different breeding compatibilities allowed me to establish a meaningful range of genetic distances that correspond with either isolation or interbreeding.

Materials and Methods

Sample collection

Samples were collected from two geographical localities: Trunk Reef (18°24' S, 146°49' E) in the Central Region of the Great Barrier Reef, and the Dampier Archipelago (20°32' S, 116°38' E) off Western Australia. On Trunk Reef, samples from 40 colonies of *A. cytherea* and *A. hyacinthus* were collected from one site of approximately 500 m^2; the low density of *A. tenuis* at this site required additional collecting at a site 500 m distant. Due to the low density of both *A. cytherea* and *A. hyacinthus* at Dampier, it was necessary to sample these species at more than one site. Tissue samples were collected by snapping off pieces 10 cm in length from individual colonies. These pieces were maintained in seawater for a maximum of 1 hr before a sub-sample was stored in liquid nitrogen. The remaining portions of the samples were bleached and dried to verify identification; these skeletons were compared with the coral collection of The Museum of Tropical Queensland (Townsville, Australia) and their identification confirmed by C.C. Wallace and J. Wolstenholme.

Electrophoresis

Tissue extracts were prepared by crushing samples of frozen coral in an equal volume of an extract solution (10 g sucrose, 0.1 ml 2-mercaptoethanol/100 ml distilled water) (modified from Willis and Ayre 1985). I used a wick impregnated in bromophenol blue every tenth lane to mark and follow the electrophoretic front. For all species, I initially screened variation at 13 enzyme systems suggested by D. Ayre, and selected eight that gave me consistently scoreable variation: Glucose-6-phosphate isomerase (*Gpi*; E.C. 5.3.1.9) and Hexokinase (*Hk*; E.C. 2.7.1.1) both run on Tris-borate buffer pH 8.4 (TEB); and Malate dehydrogenase (*Mdh*; E.C. 1.1.1.37), Malic enzyme (*Me*; E.C. 1.1.1.40), Phosphoglucomutase (*Pgm*; E.C. 5.4.2.2), Carboxylesterase (*FL-EST*; E.C. 3.1.1.1), and two Peptidases with leucylglycylglycine and valylleucine substrates (*Lgg* and *Vl*; E.C. 3.4.11/13) all run on Tris-malate buffer pH 7.4 (TM7.4). I used 12 % (w/v) horizontal starch gels. Assay conditions followed Ballment et al. (1997). Between two and five alleles were detected per

locus and these were described by the ratio of their electrophoretic mobility relative to that of the most common allele. Samples of all species were run on each gel and electromorphs with the same mobility were assumed to be homologous across species.

Data Analyses

To test the degree of panmixia within populations, departures of genotypic frequencies from those expected under Hardy-Weinberg equilibrium were estimated, using the conventional Monte Carlo Method in TFPGA (Miller 1997). The Bonferroni correction for multiple comparisons was applied for an α level of 0.05. Independence of the loci was estimated by the exact probability of linkage disequilibrium for all possible pairs of loci in each population of each species in GDA (Lewis and Zaykin 2000). Theta (θ) values (Weir and Cockerham 1984) were calculated as estimators of Wright's F_{st} statistics to determine the degree of differentiation among populations using TFPGA (Miller 1997). Probabilities for significant values of *Fst* > 0 were determined by chi-square tests across loci and 95% confidence intervals were generated by bootstrapping. A dendrogram, based on Nei's genetic distance (1978) was constructed to visualize relationships among populations of the different species. To examine any grouping in the data, a Categorical Principal Component Analysis (CatPCA) was performed in SPSS 10.0.5, using genotypes as multiple nominal variables and object principal normalization.

Results

No fixed gene differences were observed among populations of *A. cytherea* and *A. hyacinthus* (Table 3.1). In contrast, *A. tenuis* had very distinctive electromorphs (except for *Lgg*). *Me* did not present scoreable genotypes for *A. tenuis*. The frequency of polymorphic loci was high, although the number of alleles per locus was relatively low (Table 3.2). Significant deviations from H-W equilibrium were noted for some locus-population combinations (Table 3.3). These deviations were mainly due to heterozygote deficits and were correlated with significant inbreeding coefficients (F_{is}) (Table 3.2). In the case of *Me*, the

number of heterozygotes may have been underestimated due to the fact that the electrophoresis conditions may not have been optimal to resolve small differences in migration of alleles. However, heterozygote deficits that cause deviations from Hardy-Weinberg (H-W) expectations and large inbreeding coefficients (F_{is}) are a common feature of coral populations and have been reported previously for *A. cytherea* and *A. hyacinthus* (Ayre and Hughes 2000). It is unlikely that asexual reproduction is responsible for heterozygote deficits observed in this study, because I found only one multi-locus genotype repeated once. Alternatively, there is some evidence of stochastic effects, which may be artefacts of sample sizes / locus specific effects.

The analysis of independence of loci indicated that among 196 pairwise comparisons, 37 were significantly non-independent (Table 3.4). *Me* was the locus that showed the largest number of associations, however its exclusion did not appear to significantly affect *F*-statistic estimates (data not shown).

Pairwise Nei's genetic distances and F_{st} values among populations of *A. cytherea* and *A. hyacinthus* were very small (Table 3.5). Genetic differentiation between sympatric populations of *A. cytherea* and *A. hyacinthus* was significantly larger than that between allopatric populations within each species (Table 3.5, Fig 3.1). *A. tenuis* was clearly distinct from the other two species, and significant genetic differences were also observed between allopatric populations of this species. The Categorical PCA only grouped apart the two *A. tenuis* populations from the rest with the first principal component explaining 95% of the variation in the data (Fig 3.2). The analysis was not capable of discerning any other groups, even when *A. tenuis* was excluded (not shown).

Discussion

A. hyacinthus *and* A. cytherea *are distinct genetic entities*

Allozyme electrophoresis detected very low, but significant, levels of genetic differentiation between *A. cytherea* and *A. hyacinthus* in sympatry, whereas conspecific populations from distant geographic locations (i.e. eastern versus western Australia) could not be distinguished. These results suggest that hybridization occurs at a very low frequency, i.e. introgression is lower than

intraspecific gene flow between widely separated populations, thus the species are unlikely to be merging. The same pattern was observed when a comparable published allozyme data set (Ayre and Hughes 2000) was reanalyzed (Fig. 3.3). The published work examined variation at four loci in *A. cytherea* and *A. hyacinthus* populations from three different localities along the GBR, but in this case the data were used for intraspecific comparisons only. For reanalysis, I assumed that alleles with very similar reported electrophoretic mobilities were homologous between species. Note that the estimates of genetic distance and F_{st} values obtained for the published data set were larger than those obtained in my study, probably because Ayre and Hughes used only four highly polymorphic loci and because my scoring was more conservative. Unfortunately, these differences in scoring methods made it impossible to combine the two data sets.

Comparison between allopatric and sympatric populations provided a reference framework to assess the importance of low levels of genetic differentiation. The low values of Nei's genetic distance between *A. hyacinthus* and *A. cytherea* reported here are very similar to those between the Indo-Pacific *Platygyra* species (Miller and Benzie 1997), mass-spawning coral species that are also known to hybridize efficiently *in vitro* (Miller and Babcock 1997). However, in the case of *Platygyra*, no comparison was made between sympatric and allopatric populations, thus the possibility remains that the loci examined were not sufficiently variable to detect subtle differences between recently speciated taxa.

Incipient species or secondary contact?

There is no fossil record for either *A. cytherea* or *A. hyacinthus* prior to the Pleistocene (Wallace 1999), indicating that they may be relatively recent species. Their low levels of genetic differentiation are consistent with them being incipient species with incomplete reproductive barriers and retention of ancestral polymorphisms, rather than being the result of hybridization and introgression (i.e. interspecific gene flow due to secondary contact). However, coalescence times are predicted to be short in *Acropora* species because effective population sizes are likely to be small (Chapter 4), hence fixed allelic

differences are predicted to result from even relatively short (on geological time scales) periods of isolation. Thus, it is unlikely that their low genetic differentiation is solely a result of incipient species status.

Amongst Indo-Pacific species of *Acropora*, temporal differences in spawning time appear to be the major determinant of genetic distinctness (van Oppen et al., 2001; van Oppen et al., submitted), with simultaneously spawning species generally being poly- or paraphyletic. *A. cytherea* and *A. hyacinthus* frequently occur in sympatry, spawn simultaneously and are highly cross-fertile (Willis et al. 1997). Thus, introgression is likely to continuously retard lineage sorting and could account for their extremely low genetic differentiation. In summary, I favour the introgression hypothesis, although retention of ancestral polymorphisms cannot be excluded, and is likely to contribute to the complex patterns of allele sharing that appear to typify the genus.

Larval competence and allopatric genetic differences within species

The genetic differentiation between east and west Australian populations of *A. tenuis,* in contrast to the lack thereof between similar populations of *A. hyacinthus* and *A. cytherea,* may be related to differences in larval competency periods. Larval competency correlates with dispersal capacity in *Acropora*, as patterns of geographic distribution indicate that species found in remote locations have longer larval competency periods (Baird 1998). Under laboratory conditions, *A. tenuis* had a much shorter larval competency period (<20 days) in comparison to *A. hyacinthus* (up to 90 days), although no data are available for *A. cytherea* (Harrison et al. 1984, P. L. Harrison pers. comm.). Allozyme patterns indicative of high connectivity between the GBR and Western Australia have also been found for other marine organisms (e.g. *Linckia laevigata* and *Acanthaster planci*) and are thought to be related to larval transport by present day current flows and island hopping or to past dispersal events (reviewed in Benzie 1999).

Conclusions

In conclusion, despite high cross-fertility *in vitro*, natural populations of *A. cytherea* and *A. hyacinthus* are genetically distinct. The allozyme data reported here are consistent with Nested Clade Analysis of nuclear intron sequences, which indicate that *A. cytherea* and *A. hyacinthus* are distinct evolutionary lineages (Chapter 4). These results indicate that cross-fertilization trials overestimate rates of hybridization occurring under natural conditions. Cross-fertilization trials usually involve the use of sperm from only one species with eggs from another (Miller and Babcock 1997; Szmant et al. 1997; Willis et al. 1997; Hatta et al. 1999). However, during mass spawning events, eggs are generally exposed to complex mixtures of con- and heterospecific sperm, and in these situations, factors such as gamete competition are likely to modify fertilization rates. Even if moderate levels of hybridization could occur naturally, it has been suggested that disruptive selection could maintain ecologically differentiated coral morphotypes (Willis et al. 1997), while certain levels of assortative mating could maintain these morphotypes genetically distinct (Dieckmann and Doebeli 1999). Further work should address the possible role of sperm competition as a reproductive barrier in acroporid corals and examine ecological differences between cross-fertile species.

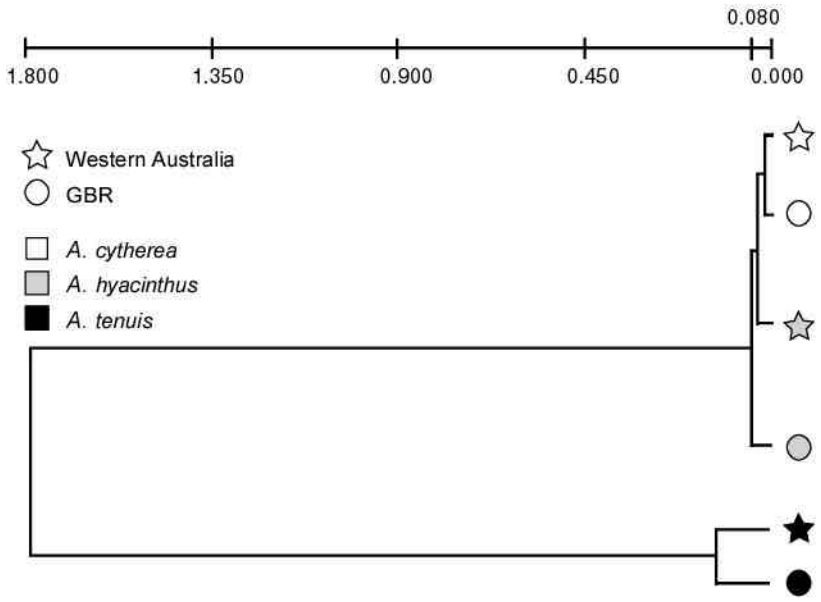


Figure 3.1 Dendrogram (UPGMA) of genetic distances (Nei, 1978) between sympatric and allopatric populations of *Acropora cytherea*, *A. hyacinthus*, and *A. tenuis* in the Great Barrier Reef and Western Australia.

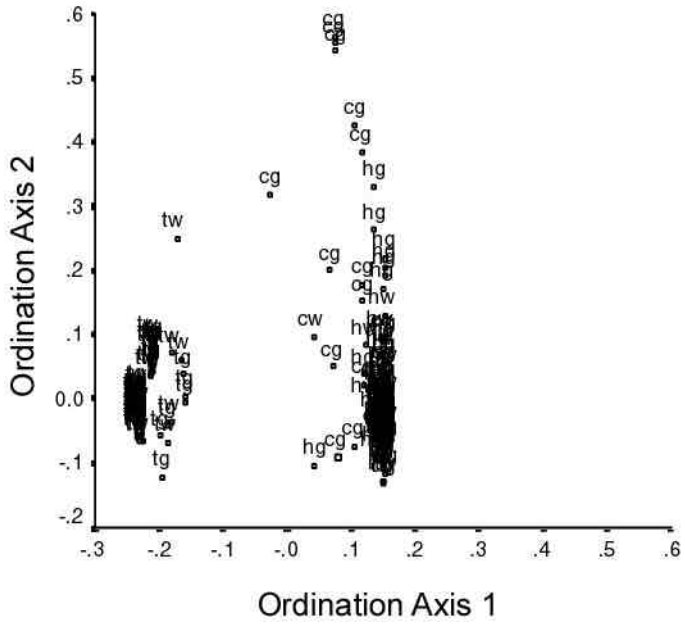


Figure 3.2 Ordination produced by the categorical principal components analysis of genotypic data of sympatric and allopatric populations of *Acropora cytherea* (c), *A. hyacinthus* (h), and *A. tenuis* (t) in the Great Barrier Reef (g) and Western Australia (w).

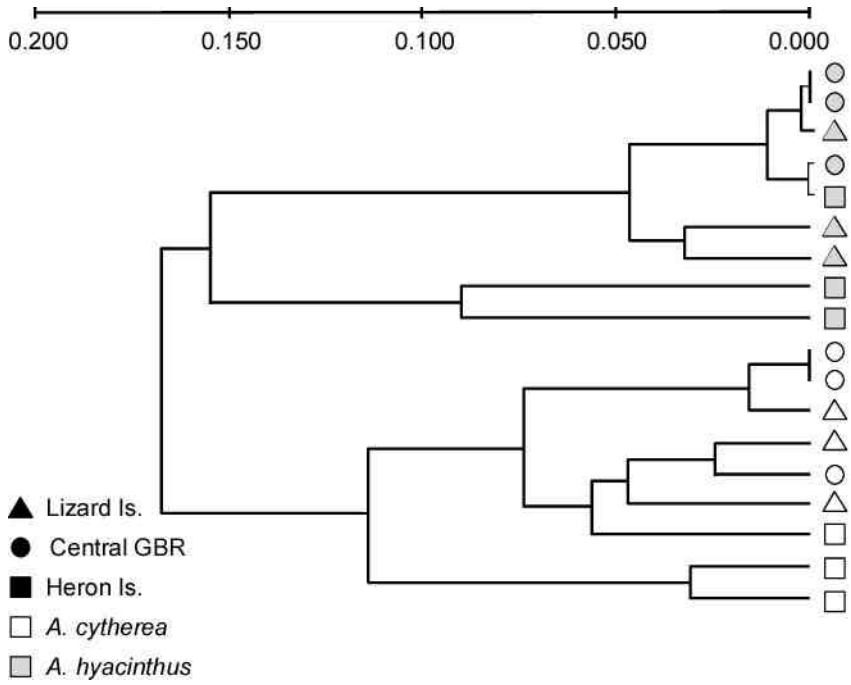


Figure 3.3 Dendrogram (UPGMA) of genetic distances (Nei, 1978) between populations of *Acropora cytherea* and *A. hyacinthus* in the Great Barrier Reef, reanalyzed from Ayre and Hughes (2000).

Table 3.1 Number of samples analyzed (N) and allele frequencies at eight allozyme loci in the acroporid corals *Acropora* cytherea **cyt***, A. hyacinthus* **hya***,* and *A. tenuis* **ten**. Population codes used: W= Western Australia, G= GBR, cyt= *A. cytherea,* hya= *A. hyacinthus*, ten= *A. tenuis.* Enzyme abreviations: Mdh = Malate dehydrogenase, Me = Malic enzyme, Pgm = Phosphoglucomutase, Hk = Hexokinase, Gpi = Glucose-6-phosphate isomerase, Vl = Peptidase with valylleucine substrate, Lgg = Peptidase with leucylglycylglycine substrate, Fl-Est = Carboxylesterase.

Locus	R.M.	Wten	Wcyt	Whya	Gcyt	Ghya	Gten
Mdh	0.65	0.04	----	----	----	----	0.26
	0.85	----	0.36	0.26	0.29	0.14	----
	1.03	0.19	----	----	----	----	----
	1.00	----	0.44	0.59	0.60	0.62	----
	1.15	----	0.20	0.15	0.11	0.24	----
	1.20	0.77	----	----	----	----	0.74
	N	51	25	40	40	38	44
Me	0.20	NA	0.08	0.07	0.11	0.34	NA
	1.00	NA	0.40	0.44	0.56	0.46	NA
	1.80	NA	0.48	0.49	0.33	0.20	NA
	2.40	NA	0.04	----	----	----	NA
	N		24	41	40	37	NA
Pgm	0.71	----	----	0.01	----	----	----
	0.78	0.01	----	----	----	----	----
	0.81	0.52	----	----	----	----	0.60
	0.84	----	0.18	0.21	0.29	0.22	----
	1.00	0.40	0.42	0.52	0.46	0.53	0.40
	1.16	----	0.38	0.23	0.24	0.22	----
	1.18	0.07	----	----	----	----	----
	1.31	----	0.02	0.02	0.01	0.03	----
	N	51	25	41	36	36	44
Hk	0.71	----	----	0.01	----	0.11	----
	0.76	0.50	----	----	----	----	0.32
	0.82	----	0.16	0.23	0.22	0.09	----
	1.00	----	0.72	0.46	0.74	0.38	----
	1.05	0.40	----	----	----	----	0.68
	1.23	----	0.10	0.30	0.04	0.42	----
	1.35	0.10	----	----	----	----	----
	1.47	----	0.02	----	----	----	----
	N	52	25	37	37	38	44

Table 3.1 Continued.

Locus	R.M.	Wten	Wcyt	Whya	Gcyt	Ghya	Gten
Gpi	0.22	0.62	----	----	----	----	----
	0.11	----	----	0.04	0.02	0.03	----
	-0.22	----	0.10	0.20	0.15	0.33	----
	-0.33	0.24	----	----	----	----	0.76
	-0.67	----	0.28	0.36	0.28	0.30	----
	-0.89	0.14	----	----	----	----	0.24
	-1.00	----	0.62	0.40	0.55	0.33	----
	-1.11	----	----	----	----	0.01	----
	N	52	25	40	41	38	41
Lgg	0.78	----	0.02	----	----	----	----
	0.89	0.10	0.26	0.10	0.18	0.08	0.17
	1.00	0.90	0.68	0.79	0.80	0.81	0.67
	1.11	----	0.04	0.11	0.03	0.11	0.17
	N	5	25	41	40	37	6
Vl	0.50	0.20	----	----	----	----	0.34
	0.58	----	----	----	0.01	0.01	----
	0.67	0.75	----	----	----	----	0.66
	0.75	----	0.21	0.01	0.12	0.42	----
	1.00	----	0.71	0.84	0.55	0.54	----
	1.08	0.05	----	----	----	----	----
	1.25	----	0.08	0.15	0.32	0.03	----
Fl.Est	N	48	24	40	39	37	43
	0.94	----	0.02	0.01	0.01	0.03	----
	1.00	----	0.94	0.99	0.97	0.95	----
	1.03	0.08	----	----	----	----	0.33
	1.11	----	0.04	----	0.01	0.03	----
	1.14	0.74	----	----	----	----	0.67
	1.28	0.18	----	----	----	----	----
	N	48	24	41	34	37	32

Table 3.2 Descriptive statistics for genetic variability in populations of three *Acropora* species. * $P<0.05$, Ho: $F_{is} = 0$. Population codes as in Table 3.1.

Population	Mean sample size per locus	Frequency of polymorphic loci	Mean no. of alleles per locus	HW expected heterozygosity	Direct count heterozygosity	F_{is}
Wten	43.86	1.00	3.00	0.4410	0.3981	0.0992
Wcyt	24.63	1.00	3.50	0.4971	0.4092	0.1799
Whya	40.13	1.00	3.38	0.4709	0.3314	0.2988*
Gcyt	38.38	1.00	3.38	0.4714	0.3468	0.2670*
Ghya	37.38	1.00	3.63	0.5199	0.4017	0.2297*
Gten	36.29	1.00	3.00	0.4775	0.4642	0.0252
Mean	36.78	1.00	3.32	0.4796	0.3919	0.1014

Table 3.3 D-values [(Ho-He)/He] indicating heterozygote deficit (negative numbers) or excess (positive numbers) for each locus and population of *Acropora cytherea, A. hyacinthus* and *A. tenuis.* Population codes as in Table 3.1. Bold numbers indicate genotypic frequencies deviating significantly ($p<0.05$) from Hardy-Weinberg expectations after Bonferroni correction for multiple tests (new $p< 0.001$). Empty cells indicate no data available.

	MDH	ME	PGM	HK	GPI	LGG	VL	FL-EST
Wten	0.1204		**0.5157**	-0.0439	**-0.6491**	0.0000	-0.2704	-0.3537
Wcyt	-0.1382	**-0.5280**	0.3342	-0.1203	-0.2564	**-0.5812**	**-0.0891**	0.0292
Whya	**-0.5180**	**-0.6155**	0.1133	**-0.4236**	0.1101	**-0.6559**	-0.3737	0.0000
Gcyt	-0.1842	**-0.4323**	0.0975	**-0.3989**	**-0.3555**	-0.3992	-0.3452	0.0075
Ghya	-0.0871	**-0.5372**	0.2773	**-0.6051**	0.0145	**-0.3423**	-0.2172	**-1.0000**
Gten	-0.0377		0.1840	-0.0705	-0.0328	0.2222	-0.2727	-0.2635

Table 3.4 Linkage disequilibrium among loci. Each symbol represents a comparison within each of the six populations in the order they appear in Table 1. 0 no linkage; - negatively correlated; + positively correlated.

	ME	PGM	HK	GPI	LGG	VL	FL-EST
MDH	000000	-00000	0000-0	+00000	0+0000	000000	000000
ME		000000	000-+0	000-+0	0+-++0	00-+00	00+-+0
PGM			000000	+00000	0+0000	+00000	0000+0
HK				-0++-0	00-0-0	000000	0000+0
GPI					000-00	-000+0	-000-0
LGG						0+0000	00+000
VL							+00000

Table 3.5 Pairwise genetic differentiation among populations of three *Acropora* species. Nei's (1978) distance below diagonal, F_{st} above diagonal. * p<0.05, Ho: F_{st} = 0. Population codes as in Table 3.1.

	Wcyt	Whya	WAten	Gcyt	Ghya	Gten
Wcyt		**0.025***	**0.443***	0.013	**0.068***	**0.444***
Whya	0.028		**0.454***	0.032	0.047	**0.458***
Wten	1.778	1.674		**0.452***	**0.427***	**0.100***
Gcyt	0.015	0.033	1.654		**0.067***	**0.457***
Ghya	0.084	0.052	1.610	0.076		**0.430***
Gten	1.981	1.891	0.131	1.884	1.831	

Chapter 4 The highly cross-fertile coral species *Acropora hyacinthus* and *A. cytherea* constitute distinct evolutionary lineages

Abstract

A major challenge for understanding the evolutionary genetics of mass spawning corals is to explain the maintenance of discrete morphospecies in view of high rates of interspecific fertilization *in vitro* and non-monophyletic patterns in molecular phylogenies. In this study, I focused on *Acropora cytherea* and *A. hyacinthus*, which have one of the highest potentials for interspecific fertilization. Using sequences of a nuclear intron, I performed phylogenetic and Nested Clade Analyses (NCA). Both species were polyphyletic in the molecular phylogeny, but the NCA indicated that they constitute different evolutionary lineages. Phylogenetic analysis using an intergenic region of the mtDNA, was inconclusive due to low levels of variability in this marker. The position of these two species differed between the nDNA and mtDNA phylogenies and was also at odds with a cladistic analysis based on morphology. Although the samples were collected from very distant geographic localities around Australia, neither the phylogenetic analyses nor the NCA showed a clear geographic pattern. I conclude that despite the potential for high levels of hybridization and introgression, *A. cytherea* and *A. hyacinthus* constitute distinct lineages and their taxonomic status is consistent with the cohesion species concept.

Introduction

The uncoupling of reproductive and morphological boundaries in a wide range of cross-fertile species (reviewed in Arnold 1997), challenges morphological species concepts underpinning traditional taxonomy (e.g. corals: Wallace and Willis 1994; Miller and Babcock 1997, Willis et al. 1997). In the case of interspecific hybridization, it is not appropriate to apply either the biological species concept (Dobzhansky 1937) or its derivatives, the evolutionary (Simpson 1944) and phylogenetic (Cracraft 1983) species concepts. Templeton (2001) has proposed the operational use of the cohesion species concept (CSC) (Templeton 1989) to address issues such as polymorphisms shared between species, lineage sorting, and hybridization using objective and quantifiable criteria. The cohesion species is a population of organisms that constitute both a distinct evolutionary lineage as well as a reproductive community in either a genetic or adaptational/ecological sense (Templeton 1989, 1994). In operational terms, the null hypothesis that previous categories (i.e. morphological species) do not correspond to phylogenetic lineages must be tested. To do so, a haplotype tree obtained by maximum parsimony is converted into a hierarchical set of nested clades (Nested Clade Analysis, NCA) (Templeton et al. 1987; Templeton and Sing 1993). Then, the null hypothesis of no association between the phylogenetic structure of the haplotype tree and the prior taxonomic categories is tested by applying exact random permutation tests (Roff and Bentzen 1989) to the nested design (Templeton and Sing 1993).

Here I applied Nested Clade Analysis to *Acropora hyacinthus* and *A. cytherea* because they exhibit one of the highest rates of interspecific fertilization recorded from *in vitro* crosses (mean = 50%) (Willis et al. 1997). Therefore they constitute an upper limit for the known possible range of hybridization within the genus. I used DNA sequence analysis of two molecular markers, one nuclear single copy non-coding region, the *Pax-C* 46/47 intron, and an intergenic region of the mitochondrial DNA, the putative control region. Van Oppen et al. (2001a) used these two markers to obtain a phylogeny of a wide range of species in the genus *Acropora*, but no *Pax-C* intron sequences of *A. hyacinthus* and only two of *A. cytherea* were included. In that phylogeny, *A.*

cytherea grouped with *A. valida*, contrasting with a cladistic analysis based on morphology (Wallace 1999), where these species occupy positions in very different clades. Therefore, I constructed molecular phylogenies (in addition to the NCA) for both markers using a larger sample of both *A. cytherea* and/or *A. hyacinthus* from distant localities around Australia. With the phylogenetic analyses I aimed to: 1) clarify their position in relation to other species within the genus; 2) determine whether they form monophyletic groups; and 3) elucidate any geographic pattern in the data. Additionally, I included in the analyses some samples of *A. spicifera*, which also forms flat-topped table colonies and is only found in Western Australia. *A. spicifera* was originally included in the *A. hyacinthus* group (Veron and Wallace 1984), but has been moved to the *A. aspera group* on the basis of corallite form in a recent revision of the genus (Wallace 1999). To determine its true affinity would help to establish whether general colony morphology (tabular vs. corymbose) is more indicative of phylogenetic relationships than corallite form (labellate round lip vs labellate flat lip).

Materials and Methods

Sample collection

Tissue samples were collected by snapping off small (2–5-cm) branches from individual colonies and placing then in 90%–100% EtOH, which was replaced after 1–2 weeks. Samples were collected from several localities around Australia (Table 4.1). Localities from Eastern Australia included the central region of the Great Barrier Reef (GBR), (Magnetic Island, Britomart and Trunk Reef); the Solitary Islands, off the coast of Coffs Harbour (New South Wales); and the Torres Strait (Dungeness and Cumberland Reef). From Western Australia, samples were collected from the Ningaloo Reef and the Dampier Archipielago.

DNA Extraction, PCR Conditions, Cloning and Sequencing Procedures

DNA was extracted from approximately 1 cm^3 of coral branch as described in van Oppen et al. (1999). Polymerase chain reaction (PCR) conditions, primers, cloning and sequencing were as described in van Oppen et al. (2001a), with the exception that in most cases I only sequenced a single clone of the *Pax-C* intron. I consider sequencing one clone per individual sufficient for the analyses, as sequences of different alleles of this marker within individuals fell within the same major phylogenetic clade in a previous study of *Acropora* species (van Oppen et al. 2001a).

Data Analyses

Sequences were aligned manually using Sequencher 3.0 (Gene Codes Corporation). For the phylogenetic analyses, I included sequences of a large range of species within the subgenus *Acropora*, both newly obtained (Table 4.1) and previously published (van Oppen et al. 2001a, 2001b). Evidence of recombination amongst nuclear intron sequences (including those from van Oppen et al. 2001a, 2001b) was explored using RETICULATE (Jakobsen and Esteal 1996). Pairwise sequence distances were calculated in PAUP* 4.0b2a. Analysis of the distribution of genetic variance within and amongst *A. cytherea*, *A. hyacinthus* and *A. spicifera* was done using analysis of molecular variance (AMOVA) (Excoffier et al. 1992) with Kimura Two-Parameter pairwise distance in ARLEQUIN 2.0 (Schneider et al. 2000). The likelihood ratio test implemented in the program ModelTest 3.04 (Posada and Crandall 1998) indicated that HKY85 was the best fit model of sequence evolution for both the *Pax-C* intron and the control region of the mtDNA. Hence, Maximum-likelihood (ML) analyses were performed in MOLPHY 2.3 (Adachi and Hasegawa 1996), under the HKY85 model. When the species of interest did not form monophyletic groups, the ML tree was compared with one in which monophyly was enforced while maintaining the same general topology and using the Shimodaira-Hasegawa test (SH-test) (Shimodaira and Hasegawa 1999) in the program Shtest v 1.0 (distributed by A. Rambaut at http://evolve.zoo.ox.ac.uk/software/shtests.html).

The *Pax-C* intron alignment contained several large indels that were excluded from the analysis, as it has been reported that their inclusion and

weighting does not affect the topology significantly (van Oppen et al. 2001a). For the NCA, I simply excluded the only indel present in the alignment of the *A. hyacinthus* group (529 bp). Similarly, repetitive regions within the control region of the mtDNA were also excluded from the analysis, because the processes by which these repeats evolve are not well understood (Fumagalli et al. 1996).

To test whether *A. hyacinthus*, *A. cytherea* and *A. spicifera* correspond to phylogenetic lineages, a Nested Clade Analysis (NCA) was performed on the *Pax-C* intron data, using the program TCS 1.13 (Clement et al. 2000). The program collapses sequences into haplotypes and calculates the frequencies of the haplotypes in the sample. These frequencies are used to estimate haplotype outgroup probabilities, which correlate with haplotype age. An absolute distance matrix is then calculated for all pairwise comparisons of haplotypes. The probability of parsimony [as defined in Templeton et al. (1992), equations 6, 7, and 8] is calculated for pairwise differences until the probability exceeds 0.95. The number of mutational differences associated with the probability just before this 95% cut-off is then the maximum number of mutational connections between pairs of sequences justified by the 'parsimony' criterion. Using these connections and the inferred missing intermediates, the program plots a haplotype network. A nested design is then drawn on top of the haplotype tree, using Templeton and Sing's (1993) algorithm. For that you need to nest haplotypes (0-step clades) that are in some sense evolutionary adjacent into 1-step clades (branches of the evolutionary tree), nest adjacent 1-step clades into 2-step clades, and so forth, until finally all data nests into a single clade. To test for the association among the clade categories and the taxonomic categories (the *Acropora* species), I used contingency table tests within each clade level (Templeton and Sing 1993) (e.g. for level 4, rows correspond to species and columns to clades 3-1, 3-2,…, 3-6. Each cell contains the number of haplotypes observed for each species-clade combination). Given the small sample sizes, the asymptotic property of a chi-square distribution cannot be assumed. Instead, I performed an exact test that uses the random permutation procedure of Roff and Bentzen (1989). In this procedure, a contingency chi-square statistic is calculated and the probability of observing the exact test statistic or larger is generated using a random permutation procedure that maintains the marginals but simulates the null hypothesis of no association. The

random permutation is implemented in Chiperm 1.2 (Chiperm, together with the other programs by D. Posada, Modeltest and TCS, are freely available at the Crandall lab web site at http://bioag.byu.edu/zoology/crandall_lab/programs.htm). NCA was not performed on the mtDNA intergenic region because both the number of taxa and the number of parsimony-informative sites were too small.

Results

Base Composition and Genetic Distances

Base compositions for both markers in *A. cytherea*, *A. hyacinthus* and *A. spicifera* are given in Table 4.2. They were homogeneous among the three species and similar to the ones found by van Oppen et al. (2001a).

Maximum corrected pairwise sequence distances among the three species were small, 6.28 % and 3.49 % for the *Pax-C* intron and the mtDNA intergenic region respectively. Most of the sequence variation in both markers occurred within species, although significant differences among species were found for the *Pax-C* intron (Table 4.3).

Phylogenetic and Nested Clade Analyses

Pax-C *Intron*

The length of the sequenced region of the intron varied among the three studied species from 434 to 888 bp, due to the presence of a long indel in 5 sequences of *A. hyacinthus* and one of *A. cytherea*. The complete *Pax-C* intron sequence alignment (including the sequences from van Oppen et al. 2001a) consisted of 1660 positions, of which 1333 were constant, 163 were variable but not parsimony-informative, and 163 were parsimony-informative. When only sequences of *A. cytherea*, *A. hyacinthus* and *A. spicifera* were analyzed, the number of parsimony-informative sites decreased to 28.

The program RETICULATE determines whether there exists a possible evolutionary history of the sequences in which all nucleotide changes at each

pair of parsimony informative sites can be inferred to have occurred only once, in which case the pair of sites is defined to be compatible. Repeated mutations, recombination or gene transfer produce incompatible sites. In the *Pax-C* sequences, the compatibility amongst the 163 parsimoniously informative sites was 92.9 % and only 7 sites were incompatible among most sequences. Therefore, there is no evidence for major recombination events within this region.

Figure 4.1A shows the ML tree rooted by using *Acropora* (*Isopora*) *cuneata.* The subgenus *A. Isopora* formed a sister group to *A. Acropora* in both cladistic analyses using morphology (Wallace 1999) and sequence analysis of Cytochrome b and NADH dehydrogenase subunit 2 (van Oppen et al. 1999; Fukami et al. 2000). The overall topology of the tree was similar to the one in van Oppen et al. (2001a). The three species, *A. cytherea*, *A. hyacinthus* and *A. spicifera* clustered in clade III in my analyses, however, the internal structure of this clade differed slightly to that of van Oppen et al.'s. The one specimen of *A. cytherea* grouped in subclade IIIC as in van Oppen et al.'s analyses, whereas some sequences of *A. cytherea* and *A. hyacinthus* were grouped in subclades IIIA/IIIB and IIIA/IIIC. Of the three species that are the focus of this study, only *A. spicifera* formed a monophyletic group. Sub-clade IIIA in van Oppen et al. (2001a) disappeared in my analysis and its members were divided between sub-clades IIIB and IIIC. The results of the SH-test (Shimodaira and Hasegawa 1999) (Table 4.4) support that *A. hyacinthus* and *A. cytherea* are truly non-monophyletic. I could not identify any phylogeographic pattern in the ML tree.

Figure 4.2 shows the nested clade design on top of the haplotype network, resulting from the NCA. Even when not all tip clades contained samples from a single species, the heterogeneity of taxonomic categories found within interior clades was non-randomly distributed. For example, within level 3, clade 3-3 was composed exclusively by *A. cytherea* samples, clade 3-4 contained only *A. hyacinthus* and all the *A. spicifera* were in clade 3-6. These observations are supported by the results of the exact random permutation test (Table 4.5), which indicated highly significant associations between species and clades at levels 4 and 3. In other words, subclades within these ones contain significantly higher numbers of one or another species. The significance is maintained even when *A. spicifera* is excluded from the analysis. Overall, these results indicate

that *A. spicifera A. cytherea* and *A. hyacinthus* constitute different evolutionary lineages, despite sharing some haplotypes.

mtDNA Intergenic Region

The complete mtDNA intergenic region alignment (both repeat and non-repeat regions) consisted of 1,836 positions, 110 more than in van Oppen et al. (2001a) due to an additional insert in an *A. hyacinthus* sequence from the Solitary Islands. The alignment of non-repeat regions consisted of 923 positions, of which 736 were constant, 66 were variable but parsimony-uninformative and 121 were parsimony-informative. However, when only sequences of *A. cytherea*, *A. hyacinthus* and *A. spicifera* were analyzed, the number of variable positions was reduced to 40, of which only 6 were parsimony-informative. Based on this and the results of the AMOVA, I believe that this marker lacks the appropriate level of variation to tell these species apart.

The general topology of the ML tree (Figure4.1B) was very similar to the one presented by van Oppen et al. (2001a). However, it was surprising that *A. cytherea*, *A. hyacinthus* and *A. spicifera* clustered within clade IV as a well-supported sister group to *A. aspera* and not in clade III, as was the case in the *Pax-C* phylogeny. Also surprising was the fact that *A. spicifera* did not form a monophyletic group, given that reciprocal monophyly is usually more rapidly achieved by mtDNA than nDNA, due to the 4-fold smaller effective population size of mtDNA. (Avise 1999). However, the *P* value associated with the SH-test was non-significant (Table 4.4), indicating that trees with enforced monophyly were not significantly worse than the ML tree. As in the *Pax-C* tree, I could not infer any phylogeographic pattern from the mtDNA tree.

Discussion

A. cytherea *and* A. hyacinthus *are not monophyletic*

Phylogenetic analysis of *Pax-C* intron sequences indicates that *A. cytherea* and *A. hyacinthus* are not monophyletic and thus do not conform to the

phylogenetic species concept. In contrast, the mtDNA marker does not allow rejection of monophyly for these species. The apparently contradictory results for the *Pax-C* and mtDNA phylogenies may be related to the generally low levels of variability in mtDNA compared to nuclear markers found in acroporid corals. The slow rate of evolution of coral mtDNA is believed to be associated with a mismatch repair mechanism (Pont-Kingdon et al. 1994, van Oppen et al. 1999). *A. spicifera* is monophyletic according to the *Pax-C* tree and this hypothesis could not be rejected using the mtDNA data, even when it does not form a monophyletic group in the mtDNA phylogeny. Given the incongruence between the two molecular trees, I could not establish whether *A. spicifera* has greater genetic affinity with the *A. aspera* or the *A. hyacinthus* group, and therefore I could not infer whether colony morphology or corallite form is phylogenetically more informative.

There is no geographic pattern in phylogenies

The absence of a geographic pattern in both trees correlates with the great potential for dispersal and gene flow in these species (Ayre and Hughes 2000; Chapter 3). Allozyme data for other marine organisms, such as *Linckia laevigata* and *Acanthaster planci*, also indicate a high level of connectivity between the GBR and Western Australia (reviewed in Benzie 1999), which may either be related to larval transport by present day current flows and island hopping or to past dispersal events (Benzie 1999).

A. hyacinthus *and* A. cytherea *constitute distinct evolutionary lineages*

All three species constitute different evolutionary lineages according to the NCA. Given their potential for high rates of cross-fertilization *in vitro* (Willis et al. 1997), it is difficult to explain how *A. cytherea* and *A. hyacinthus* could be maintained as distinct lineages. One possibility is that cross-fertilization trials overestimate the rate of hybridization that actually occurs under natural conditions. Cross-fertilization trials usually involve the combination of sperm from one species with eggs from another (Miller and Babcock 1997; Szmant et al. 1997; Willis et al. 1997; Hatta et al. 1999). However, during mass spawning

events the eggs are exposed to a mixture of conspecific and heterospecific sperm that could lead to gamete competition and reduced interspecific fertilization. Alternatively, *A. hyacinthus* and *A. cytherea* tend to differ in their depth distributions (LM and BW, unpublished data) supporting the idea that they are ecologically differentiated and may be under the effect of disruptive selection (Willis et al. 1997). Even when the fertilization rate between these species is very high, it is on average almost half of that within species (50% vs. 95%, interspecific vs intraspecific respectively). Unless hybrids are more fit than purebreds, in an evolutionary time scale the outcome of this difference in fertilization rates is assortative mating, which together with disruptive selection acting upon ecologically differentiated morphotypes can lead to evolutionary branching (Dieckmann and Doebeli 1999). A third possibility is that hybrids are unable to backcross with the parentals, because of chromosomal rearrangements or ecological isolation (reviewed in Riesberg 2000).

Lack of genealogical concordance between the phylogenies

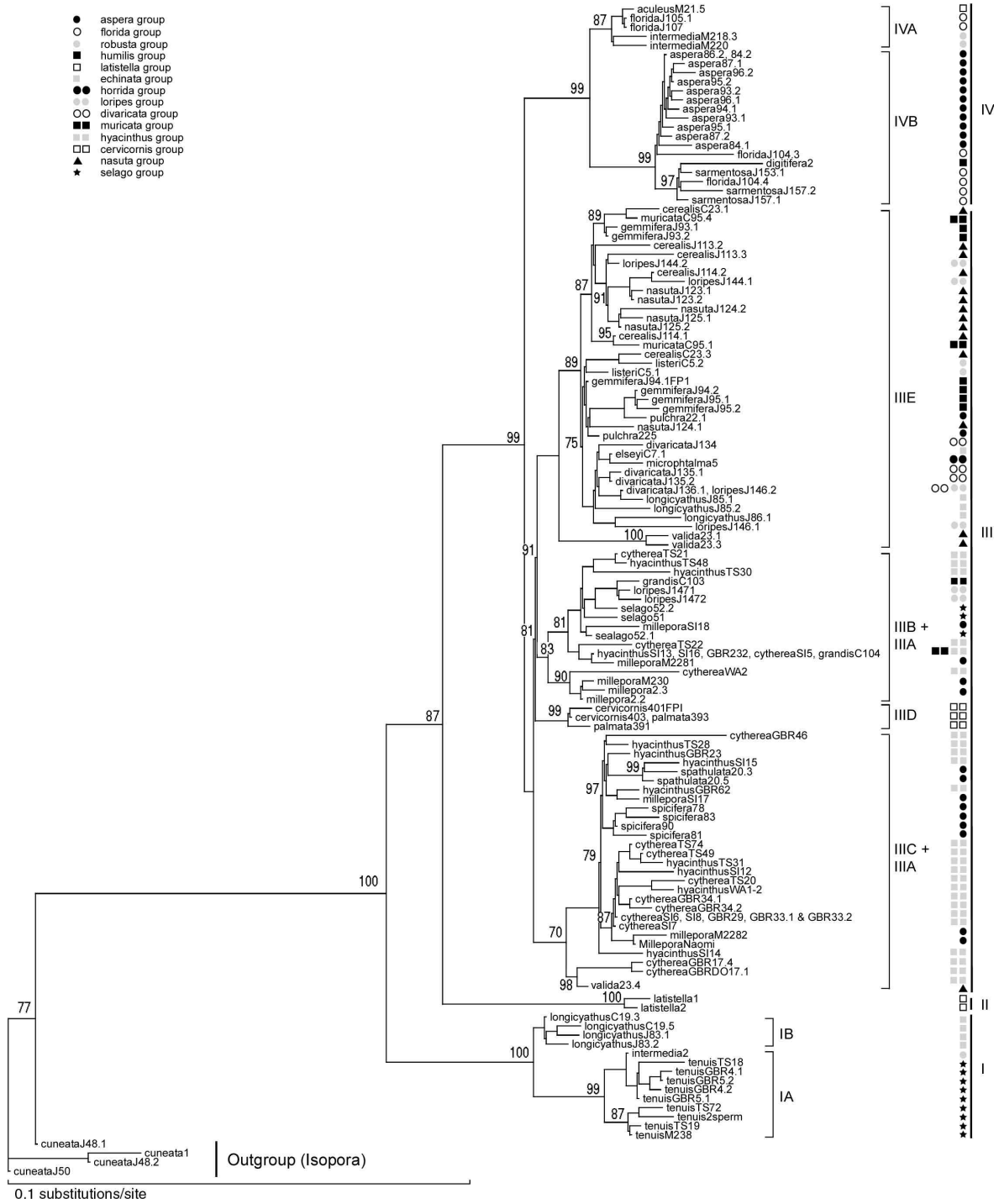
The difference in the position of *A. cytherea* and *A. hyacinthus* in the nDNA and mtDNA trees (i.e. clade III in the former and clade IV in the latter analysis) could be explained by either recent speciation coupled with incomplete lineage sorting or by introgression. Avise and Ball (1990) showed that short separation times result in a lack of lineage sorting and genealogical concordance, so that phylogenetic analyses of molecular markers represent gene genealogies rather than organismal phylogenies. Distinguishing between introgression and ancestral polymorphisms in phylogenetic analysis can be especially difficult when species are of relatively recent origin and the fossil record is scarce (Avise 1999). This is the case for species of the *A. hyacinthus* group, for which the oldest fossil is from the Pleistocene (Wallace 1999). Nevertheless, coalescence times for acroporid species should be relatively small, because, despite very large and highly connected contemporary populations, their effective population sizes are predicted to be small. This is because of extremely high variances in reproductive success expected in association with broadcast spawning (Avise 1999, p. 46), clonal population structures, and periodic mass mortality events, which all reduce long-term effective population

sizes. A short coalescence time for acroporid species would favor the introgression hypothesis. The introgression hypothesis is further supported by Templeton's (1994) argument that incomplete sorting of ancestral polymorphisms can be rejected in NCA when interspecific coalescence and sympatry are associated. In other words, hybridization is a more probable explanation when sympatric samples of different species are grouped within the same clade. In my NCA, the lack of significance within clades 3-2 and 3-5 suggest introgression, as clades 2-4 and 2-9 are composed mainly of sympatric individuals or of individuals coming from the least distant geographic localities (e.g. the grouping of GBR and Solitary Island *A. hyacinthus*). van Oppen et al. (2001a) also favored the introgression hypothesis in their analyses of acroporid phylogenies based upon evidence of high potential for cross-fertilization among many of these species (Willis et al. 1997) and the correlation of major clusters in the trees with species that are temporally reproductively isolation (i.e. differ in the timing of spawning). Even when most of my results support the introgression scenario, I have to acknowledge a couple of pitfalls such as the lack of a phylogeograhic signal in both the ML tree and NCA, and the grouping of allopatric samples of *A. cytherea* and *A. hyacinthus* in the clade 2-1 of the NCA.

It must also be noted that phylogenetic trees can change noticeably when more sequences are added. van Oppen et al.'s IIIA clade was dispersed throughout clades IIIB and IIIC in my expanded *Pax-C* analysis, suggesting that the sub-clade structure of the large clade III is fairly tenuous and that species relationships within it are fairly ill-defined. Additionally, the position of members of the *A. hyacinthus*, *A. latistella* and *A. selago* groups disagrees with Wallace's (1999) cladistic analysis based on morphology. In the morphological analysis, these groups are closely related, but in both molecular phylogenies, *A. aspera*, *A. latistella* and *A. tenuis* (a member of the *A. selago* group) form independent clades (II and IA, respectively). The clustering of these species in different clades may be explained by reproductive isolation (van Oppen et al. 2001a), given that *A. latistella* and *A. tenuis* spawn respectively two weeks out of phase and 2-3 hours earlier on the same day, than most *Acropora* species (Babcock et al. 1986). Reiterate evolution of morphological characters may also serve as a possible explanation for the incongruence between morphology and genetics.

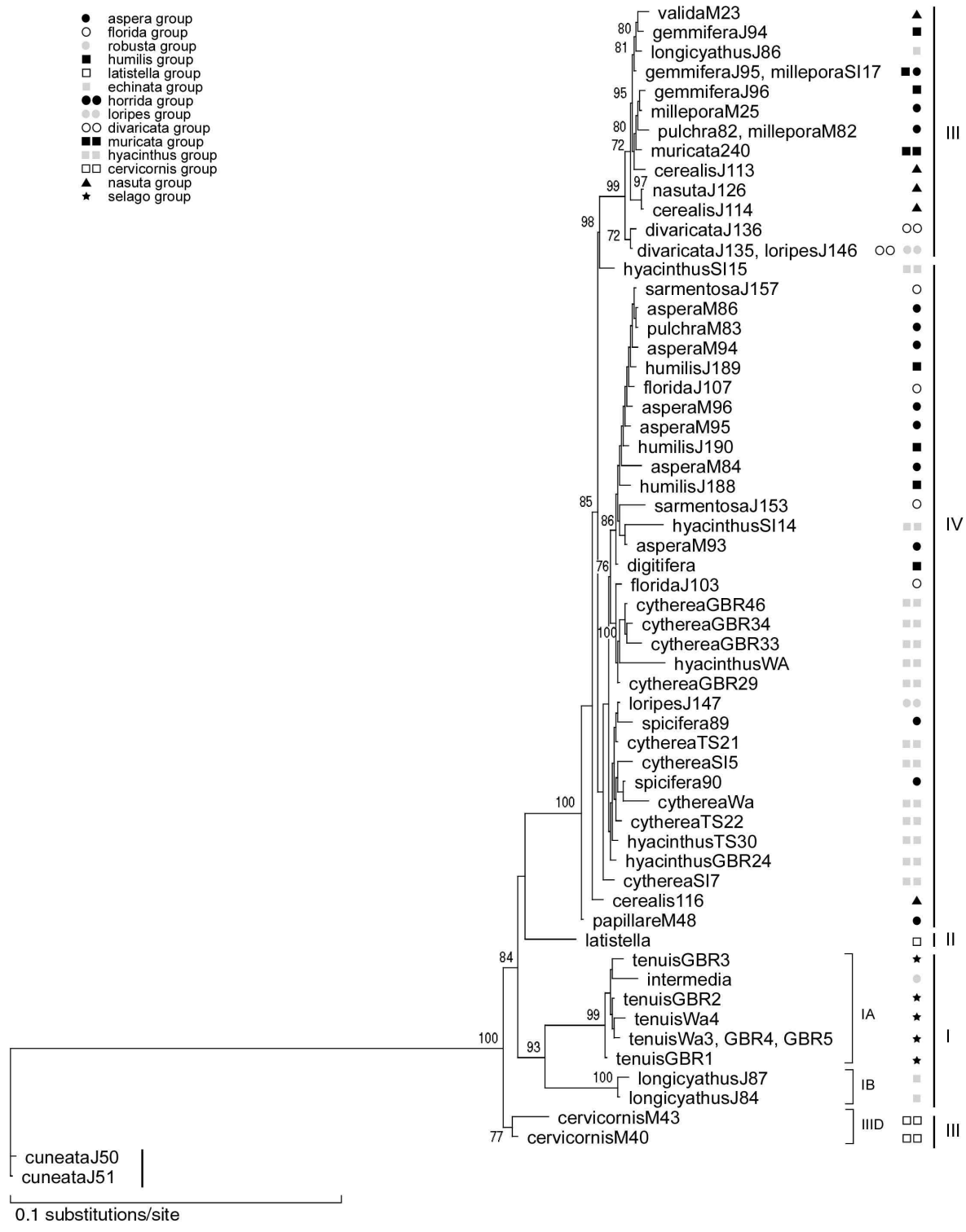
Conclusions

In summary, phylogenetic analyses suggest that *A. cytherea* and *A. hyacinthus* are part of a large syngameon, or hybridizing group of species (Grant 1957; Veron 1995). Nevertheless, the NCA indicates that either hybridization is infrequent or disruptive selection is operating to maintain these species as distinctive evolutionary lineages. I conclude that, despite showing high cross-fertility, this pair of acroporid species should not be synonimised. Changes in taxonomy must be considered very carefully, because taxonomy does not operate in an intellectual vacuum, but instead it affects other disciplines such as ecology, paleontology, biogeography and conservation (Knowlton and Weigt 1997; Knowlton 2001). For the time being, and in the case of *Acropora*, I advocate taxonomic conservatism and the use of morphological species as operational taxonomic units (Veron 1995) following the latest revision of the genus (Wallace 1999). I agree with Dupré (1999) that to celebrate every change in the consensus of phylogenetic relationships with a change in taxonomic nomenclature is an "inexcusable imposition of a particular professional perspective on the long-suffering consumers of taxonomy outside these phylogenetic debates". I look forward to the addition of new genetic markers and analyses of species representatives from other groups to further assess the evolving taxonomy of acroporid corals.


aspera group
florida group
robusta group
humilis group
latistella group
echinata group
horrida group
loripes group
divaricata group
muricata group
hyacinthus group
cervicornis group
nasuta group
selago group
IVA
IVB
IV
IIIE
III
IIIB +
IIIA
IIID
IIIC +
IIIA
II
IB
IA
I
Outgroup (Isopora)
0.1 substitutions/site

A

Figure 4.1 Rooted maximum-likelihood trees of *Acropora* species using the HKY85 model of sequence evolution for (A) the *Pax-C* intron and (B) the mtDNA intergenic region. Values on branches indicate bootstrap values (1,000 replicates). Symbols for the different species groups are given. The four major clades are indicated by I, II, III and IV. A number followed by a letter marks subclades within clades. Sample codes are as in Table 4.1, and in van Oppen et al (2001a and b). A dot and a number after the sample code indicate the clone that was sequenced. Several names separated by commas indicate samples with identical sequences. Sample names for *A. cytherea* and *A. hyacinthus* indicate geographic origin: GBR, Central Great Barrier Reef; SI, Solitary Islands; TS, Torres Strait; and WA, Western Australia.


aspera group
florida group
robusta group
humilis group
latistella group
echinata group
horrida group
loripes group
divaricata group
muricata group
hyacinthus group
cervicornis group
nasuta group
selago group
validaM23
gemmiferaJ94
longicyathusJ86
gemmiferaJ95, milleporaSI17
gemmiferaJ96
milleporaM25
pulchra82, milleporaM82
muricata240
cerealisJ113
nasutaJ126
cerealisJ114
divaricataJ136
divaricataJ135, loripesJ146
hyacinthusSI15
sarmentosaJ157
asperaM86
pulchraM83
asperaM94
humilisJ189
floridaJ107
asperaM96
asperaM95
humilisJ190
asperaM84
humilisJ188
sarmentosaJ153
hyacinthusSI14
asperaM93
digitifera
floridaJ103
cythereaGBR46
cythereaGBR34
cythereaGBR33
hyacinthusWA
cythereaGBR29
loripesJ147
spicifera89
cythereaTS21
cythereaSI5
spicifera90
cythereaWa
cythereaTS22
hyacinthusTS30
hyacinthusGBR24
cythereaSI7
cerealis116
papillareM48
latistella
tenuisGBR3
intermedia
tenuisGBR2
tenuisWa4
tenuisWa3, GBR4, GBR5
tenuisGBR1
longicyathusJ87
longicyathusJ84
cervicornisM43
cervicornisM40
cuneataJ50
cuneataJ51
III
IV
II
IA
I
IB
IIID
III
0.1 substitutions/site

B

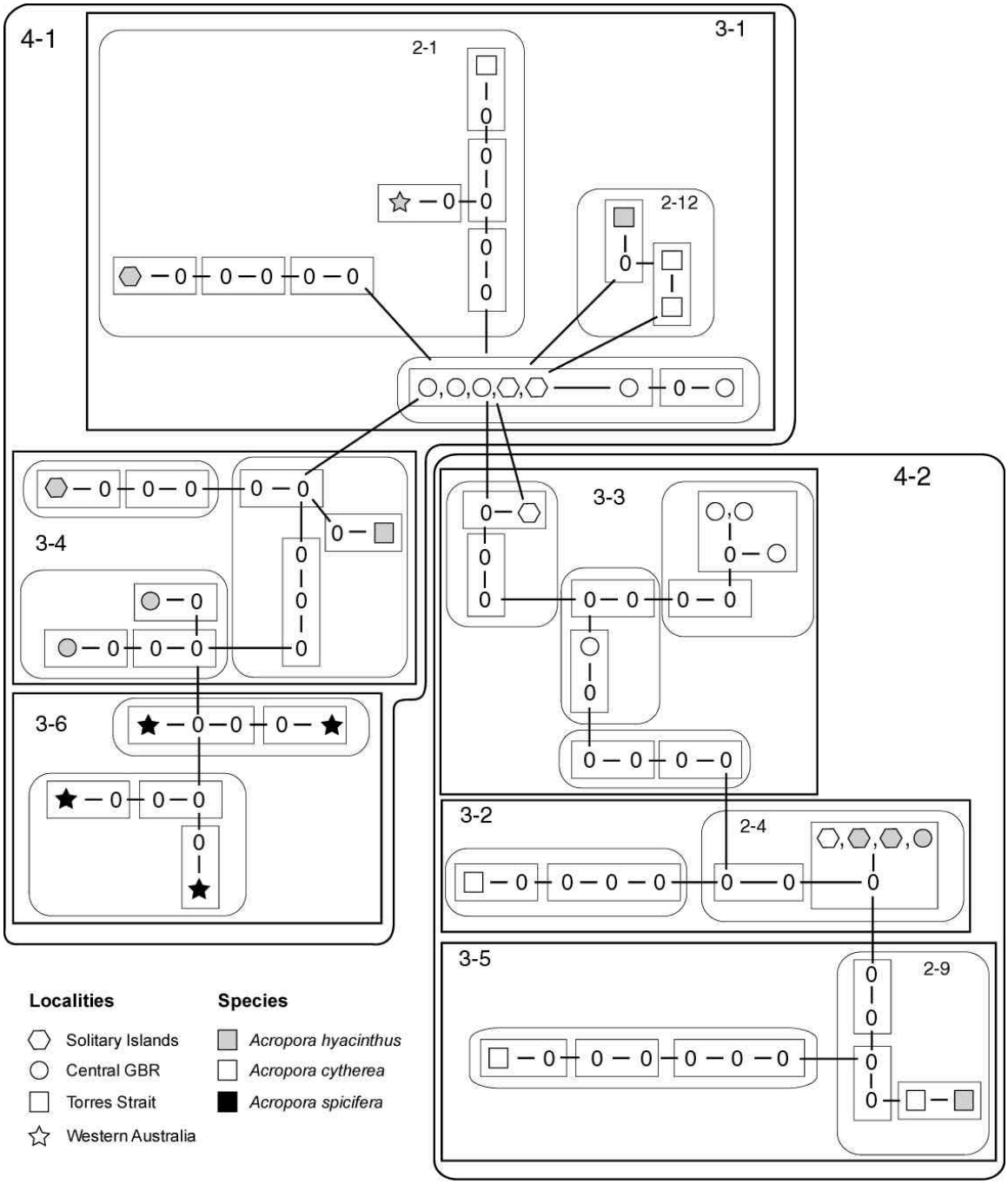

4-1
3-1
2-1
2-12
3-4
3-6
4-2
3-3
3-2
2-4
3-5
2-9
Localities
Solitary Islands
Central GBR
Torres Strait
Western Australia
Species
Acropora hyacinthus
Acropora cytherea
Acropora spicifera

Figure 4.2 Haplotype tree and nested clade design for the *Pax-C* intron of *Acropora cytherea*, *A. hyacinthus* and *A. spicifera*. An "0" represents an inferred intermediate haplotype that was not sampled. Rectangular, narrow lined boxes indicate one-step clades; narrow lined boxes with rounded corners represent two-step clades; double thick lined rectangles represent three-step clades; and double thick lined boxes with rounded corners represent four-step clades.

Table 4.1 Sample codes and collection locations

Species	Colletion Location	Sample Code	Sequences Obtained	GeneBank accession Nos
A. cytherea	Magnetic Is., GBR	cythereaGBRDO17	*Pax* I	
A. cytherea	Elizabeth, GBR	cythereaGBR46	*Pax* I, mtDNA IGR	
A. cytherea	Britomart, GBR	cythereaGBR29	*Pax* I, mtDNA IGR	
A. cytherea	Trunk, GBR	cythereaGBR33	*Pax* I, mtDNA IGR	
A. cytherea	Trunk, GBR	cythereaGBR34	*Pax* I, mtDNA IGR	
A. cytherea	Dungeness, TS	cythereaTS20	*Pax* I	
A. cytherea	Dungeness, TS	cythereaTS21	*Pax* I, mtDNA IGR	
A. cytherea	Dungeness, TS	cythereaTS22	*Pax* I, mtDNA IGR	
A. cytherea	Dungeness, TS	cythereaTS49	*Pax* I	
A. cytherea	Cumberland, TS	cythereaTS74	*Pax* I	
A. cytherea	Ningaloo, WA	cythereaWA1	mtDNA IGR	
A. cytherea	Ningaloo, WA	cythereaWA2	*Pax* I	
A. cytherea	Solitary Is., NSW	cythereaSI5	mtDNA IGR	
A. cytherea	Solitary Is., NSW	cythereaSI7	mtDNA IGR	
A. cytherea	Solitary Is., NSW	cythereaSI15	*Pax* I	
A. cytherea	Solitary Is., NSW	cythereaSI16	*Pax* I	
A. cytherea	Solitary Is., NSW	cythereaSI17	*Pax* I	
A. cytherea	Solitary Is., NSW	cythereaSI18	*Pax* I	
A. hyacinthus	Magnetic Is., GBR	hyacinthusGBR232	*Pax* I	
A. hyacinthus	Britomart, GBR	hyacinthusGBR23	*Pax* I	
A. hyacinthus	Britomart, GBR	hyacinthusGBR24	mtDNA IGR	
A. hyacinthus	Trunk, GBR	hyacinthusGBR62	*Pax* I	
A. hyacinthus	Dungeness, TS	hyacinthusTS28	*Pax* I	
A. hyacinthus	Dungeness, TS	hyacinthusTS30	*Pax* I, mtDNA IGR	
A. hyacinthus	Dungeness, TS	hyacinthusTS31	*Pax* I	
A. hyacinthus	Dungeness, TS	hyacinthusTS48	*Pax* I	
A. hyacinthus	Ningaloo, WA	hyacinthusWA1	*Pax* I, mtDNA IGR	
A. hyacinthus	Solitary Is., NSW	hyacinthusSI13	*Pax* I	
A. hyacinthus	Solitary Is., NSW	hyacinthusSI14	*Pax* I, mtDNA IGR	

Table 4.1 Continued.

Species	Colletion Location	Sample Code	Sequences Obtained	GeneBank accession Nos
A. hyacinthus	Solitary Is., NSW	hyacinthusSI15	*Pax* I, mtDNA IGR	
A. hyacinthus	Solitary Is., NSW	hyacinthusSI16	*Pax* I	
A. spicifera	Bundegi, WA	spiciferaWA78	*Pax* I	
A. spicifera	Coral Bay, WA	spiciferaWA81	*Pax* I	
A. spicifera	Coral Bay, WA	spiciferaWA83	*Pax* I	
A. spicifera	Coral Bay, WA	spiciferaWA89	mtDNA	
A. spicifera	Coral Bay, WA	spiciferaWA90	*Pax* I, mtDNA IGR	
A. millepora	Solitary Is., NSW	milleporaSI17	*Pax* I, mtDNA IGR	
A. millepora	Solitary Is., NSW	milleporaSI18	*Pax* I	
A. selago	Old, GBR	selago51	*Pax* I	
A. selago	Old, GBR	selago52	*Pax* I	
A. tenuis	Trunk, GBR	TenuisGBR4	*Pax* I, mtDNA IGR	
A. tenuis	Trunk, GBR	TenuisGBR5	*Pax* I, mtDNA IGR	
A. tenuis	Dungeness, TS	tenuisTS18	*Pax* I	
A. tenuis	Dungeness, TS	tenuisTS19	*Pax* I	
A. tenuis	Cumberland, TS	tenuisTS72	*Pax* I	
A. tenuis	Ningaloo, WA	tenuisWa3	mtDNA IGR	
A. tenuis	Ningaloo, WA	tenuisWa4	mtDNA IGR	

Table 4.2 Mean base compositions (%) and ranges of Kimura Two-Parameter pairwise sequence distances (%) for the *Pax-C* intron and the mtDNA intergenic region (nonrepeat regions) in *A. cytherea*, *A. hyacinthus* and *A. spicifera*. Standard deviations are given in parentheses.

	A	C	G	T	Distance range
Pax-C intron	32.53 (0.002)	19.97 (0.003)	18.23 (0.002)	29.27 (0.003)	0.00-6.28
MtDNA intergenic region	24.18 (0.005)	19.22 (0.003)	24.60 (0.003)	32.00 (0.007)	0.00-3.49

Table 4.3 Analysis of genetic variance of the *Pax-C* intron and an intergenic region of the mtDNA within and among *A. cytherea, A. hyacinthus and A. spicifera*, using AMOVA.

	Within Species		Among Species	
	% Variance	*P*	% Variance	*P*
Pax-C intron	85.51	<0.001	14.49	<0.001
MtDNA intergenic region	95.14	<0.001	4.86	0.190

Table 4.4 Results of Shimodaira-Hasegawa Test of *A. cytherea*, *A.hyacinthus* and *A. spicifera* monophyly for the *Pax-C* intron and mtDNA intergenic region data sets.

	-ln *L*	Difference in -ln *L*	*P*
***Pax-C* Intron**			
ML tree (best tree)	-3,465.5441		
Enforced monophyly			
A. hyacinthus	-3,562.2058	96.6617	<0.001
A. cytherea	-3630.5696	165.0255	<0.001
both spp	-3,626,4012	160.8571	0.002
mtDNA intergenic region			
ML tree (best tree)	-2,762.2849		
Enforced monophyly			
A. hyacinthus	-2,738.0678	18.4970	0.175
A. cytherea	-2,743.1011	23.4303	0.114
A. spicifera	-2,724.7783	5.2074	0.620
All spp	-2,717.7957	44.4892	0.055

Table 4.5 Nested exact contingency analysis of species with clades of the *Pax-C* intron. The nested design is given in Figure 2. A standard contingency chi-square statistic is calculated, and its exact significance is determined by 1000 random permutations that preserve the marginal values. The probability column refers to the frequency with which these randomly generated chi-square statistics were equal to or greater than the observed chi-square

Source	Chi-square statistic	*P*
4-Step clades	12.6674	0.002
3-Step clades		
Within 4-1	30.2308	<0.001
Within 4-2	4.2370	0.193
2-Step clades		
Within 3-1	5.4889	0.061
Within 3-2	1.8750	0.402
Within 3-5	0.7500	1.000
1-Step clade within 2-12	3.0000	0.338

Chapter 5 Interspecific sperm competition as a mechanism to maintain cross-fertile species as distinct lineages

Abstract

Cross-fertilization trials may overestimate the rate of hybridization that actually occurs under natural conditions, because they are non-competitive, involving the exclusive combination of sperm from one species with eggs from another. I designed breeding trials using acroporid corals to test whether the mixture of conspecific and heterospecific sperm inhibits interspecific fertilization, promoting conspecific sperm precedence. Additionally, I tested whether the mixture of self-sperm and heterospecific sperm promotes self-fertilization, a phenomenon known as "mentor effect". For paternity analysis, I intended to use existing nuclear introns as well as to develop highly-polymorphic microsatellite markers, but the development of microsatellite markers was unsuccesful. I infer that the main reason for the failure in obtaining microsatellites from acroporid corals is their scarcity, probably associated with the small size of the *Acropora* genome. Because fertilization rates between the two species crossed were below 5%, which was considered too low to be useful for proper sperm competition experiments, I did not carry out paternity analysis. The following trends were observed in the fertilization data alone: 1) crosses involving conspecific sperm resulted in high fertilization rate, independent of the proportion of conspecific vs. heterospecific sperm, suggesting that heterospecific sperm do not adversely affect conspecific fertilization, as is the case in ascidians; and 2) the fertilization rate using mixtures of self and heterospecific sperm were equal or smaller than using self-sperm alone, therefore there is no evidence of a "mentor effect" in *Acropora*.

Introduction

It is not known how highly cross-fertile *Acropora* species are being maintained as distinct lineages through evolutionary time. One possibility is that cross-fertilization trials overestimate the rate of hybridization that actually occurs under natural conditions, a common observation in the study of other hybridizing species (reviewed in Arnold 1997). In corals, cross-fertilization trials indicate high breeding compatibilities among several species, but they usually involve the exclusive combination of sperm from one species with eggs from another (Miller and Babcock 1997; Szmant et al. 1997; Willis et al. 1997; Hatta et al. 1999). Nevertheless, during mass spawning events eggs are exposed to a mixture of conspecific and heterospecific sperm that could lead to gamete competition and reduced interspecific fertilization. Willis and Ayre (unpublished data) found that in sperm competition trials between *A. millepora* and *A. pulchra*, conspecific sperm sired most of the embryos in the majority of cases. In two out of 15 trials, however, all embryos were found to be hybrids. Thus, in at least some cases, *in vitro* hybridization may occur in the presence of conspecific sperm in this species group.

The presence of heterospecific sperm may also cause disruption of the self-incompatibility system and enhance the incidence of self-fertilization, a phenomenon called 'mentor effect' that has been shown to occur in other taxa (reviewed in Arnold 1997). Laboratory experiments with gametes from the same and different coral colonies show that a range of capabilities for self-fertilization exists, from completely self-sterile colonies in species of *Montipora*, through extremely low incidence of self-fertilization in species of *Acropora*, to partially self-fertile colonies of *Goniastrea favulus* and *G. aspera* (Heyward and Babcock 1986; Harrison and Wallace 1990; Willis et al. 1997). However, as in the case of the hybridization experiments, these self-compatibility experiments were non-competitive, involving only eggs and sperm from the same colony in each trial.

The gamete-competition experiments by Ayre and Willis involved the use of allozymes to identify individuals used in the experimental crosses. The use of allozymes required genotyping a large number of individuals to obtain homozygotes for the crosses. Moreover, recruits needed to be reared for 6 days

to obtain sufficiently high expression levels of the allozymes. These factors limited the number of crosses that could be performed in a single breeding season. The use of DNA molecular markers to estimate paternity in sperm competition crosses may help to overcome the problems associated with the use of allozymes. I intended to use two types of DNA markers for genotyping: 1) microsatellites, which are DNA sequences consisting of short (1-6 bp), tandomly repeated motifs with high variability in repeat size (see Goldstein and Schlötterer 1999); microsatellites usually possess more than ten alleles per locus and heterozygosities above 0.6 (Bowcock et al. 1994); and 2) single copy nuclear introns, which, being non-coding regions, tend to accumulate mutations relatively rapidly. Such mutations can in most cases be easily detected without the need of sequencing by single stranded conformation polymorphism (SSCP) (Sunnucks et al. 2000). DNA markers can be amplified from the tiny amounts of DNA contained in embryos using PCR.

I designed breeding trials using acroporid corals in order to test whether: 1) the presence of both conspecific and heterospecific sperm inhibits interspecific fertilization, promoting conspecific sperm precedence; 2) the mixture of self-sperm and heterospecific sperm promotes self-fertilization (i.e. 'mentor effect'); 3) conspecific sperm inhibit self-fertilization (as a mechanism to minimise inbreeding).

Materials and Methods

Coral collections

During the December 1999 spawning season on the GBR, six coral colonies of each of the two species *Acropora hyacinthus* and *A. cytherea* were collected from SE Pelorus and NE Reef on Orpheus Island (18°34.3' S, 146°29.5' E). During the March 2000 spawning season in Coral Bay (23 09' S, 113 46' E), Western Australia, three colonies of *A. nasuta* and one of *A. cerealis* were collected instead of colonies of *A. hyacinthus* and *A. cytherea*, because the latter was not found at this locality.

Gamete preparation

Gravid coral colonies were collected the afternoon prior to the mass spawning and placed into separate plastic containers. Gamete preparation followed Willis et al. (1997).

Fertilization trials

Treatments to test for sperm competition are described in Table 5.1. All crosses were performed in 25 ml screw-cap glass vials. Sperm were added to the vials and mixed prior to adding slightly more than a hundred eggs per vial. Each cross was replicated four times in separate vials. Vials were incubated by placing them in mesh bags and either holding them in a tank with circulating sea water (Orpheus Island Research Station) or tying them to a boat and letting them float at sea (Coral Bay). Three vials of each cross were fixed with formalin-β-glycerophosphate six hours after having added the sperm and embryos were examined under a dissecting microscope. The fourth vial of each cross was drained and the eggs and embryos preserved in 70% ethanol for DNA extractions.

Development of microsatellites by affinity chromatography

To construct a genomic library of *Acropora millepora* enriched with microsatellites, I used a Sepharose-DNA activated column to select restriction fragments containing microsatellites. The DNA was extracted from sperm and a rough equivalent of 5.8 x 10^6 genomes was digested using *Sau3A*I and *Mbo*I. As recovery from the affinity column is typically low, SAUL adapters were ligated to the fragments in order to provide anchors for amplifying eluted clones (Strassman et al. 1996). Preparation of the column and elution followed Brenig and Brem (1991) with 10 µM 30mer oligos (AGTG, TAG, ACA, AG and TA) coupled to activated Sepharose. Initial cloning into *BamH*I digested p-Bluescript showed a preference for very small inserts and so the SAUL overhangs were filled in and the inserts cloned into pGEM-T.

Around eight hundred clones were manually dot blotted to nylon membranes. Screening with end labeled probes $(TA)_{30}$, $(AG)_{30}$ and $(ACA)_{30}$ yielded more

than a hundred positive clones, despite washing with very high stringency (60º C, 0.5X SSPE). Upon sequencing 16 random positives, 13 were found to consist of adapter concatemers (each pair of adapters typically being interrupted by 5 to 8 bp of random sequence), while the remaining three were *de novo* sequences lacking microsatellites.

DNA extractions and amplification from single embryos.

Single copy genes were successfully amplified from single embryos by extracting the DNA using the GenomicPrep Cell and Tissue DNA Extraction Kit (Amersham Pharmacia Biotech Inc.), following the instructions of the manufacturers and adding 20 µl of 20 mg/ml proteinase K during the incubation step. Nevertheless, PCR profiles required 35 cycles, rather than the usual 30.

Determination of paternity using one microsatellite and two nuclear introns

Given that the microsatellite development was unsuccessful, I assayed the following alternative markers in order to estimate paternity of coral embryos: 1) AFO 53, a microsatellite developed by Dr. A. Chen (Academia Sinica, Taiwan) from a genomic library of *Acropora formosa*; 2) the second intron of the mini collagen gene (Wang et al 1995; Hatta et al. 1999); and 3) the intron of cnox2Am (Hayward et al. 2001). The list of primers for these markers and their melting temperatures, as well as the length of the amplified regions are shown in Table 5.2. PCR products corresponding to the microsatellite in the parentals were run on a 5% 19:1 acrylamide:bis-acrylamide denaturing gel using Hex-labeled primers and visualized using the Gel-Scan2000 system (Corbett Research). The single copy nuclear markers were labeled and visualized in a similar fashion but run on 4% 37:1 acrylamide:bis-acrylamide non-denaturing gels in order to observe SSCP patterns (Sunnucks et al. 2000).

Results

Spawning failure at Orpheus Island

All the 1999 crosses at Orpheus Island failed. Of more than six gravid colonies of each species, only three (one *A. cytherea* and two *A. hyacinthus*) released gametes. Very few gametes were released and they were not viable, as asserted by the lack of fertilization in a conspecific control cross between the two *A. hyacinthus* colonies. This happened despite the fact that the colonies were maintained under conditions that have previously been shown optimal (Willis et al. 1997) and the colonies looked healthy (i.e. they did not bleach or release large amounts of mucus). However, weather conditions were marked by heavy rain during the predicted spawning nights associated with strong winds and low seawater temperature (26-27 °C degrees in comparison to the usual 29°C at that time of the year).

Patterns in fertilization data from the Coral Bay 2000 spawning period

Sperm competition trials involving mixtures of hetero-, self- and conspecific sperm crossed with eggs of *A. cerealis* and *A. nasuta* resulted in a very low interspecific fertilization rate (3.47 % ± 0.94, average ± standard error). However, some patterns in the fertilization data are worth mentioning.

1. Crosses involving conspecific sperm resulted in high fertilization rate, independently of the proportion of conspecific vs. heterospecific sperm (Figure 5.1).
2. Using *A. nasuta* eggs, the fertilization rate was higher using self-sperm alone than either heterospecific sperm alone or mixtures of self and heterospecific sperm (Figure 5.2).
3. The mixture of self and conspecific sperm in proportions 100:1 reduced the fertilization rate in comparison to the use of proportions 1:100 or 1:1 and in comparison to the control with conspecific sperm only (Figure 5.3).

Genotyping of Embryos

I was able to successfully amplify AFO53 as well as the mini collagen and cnox2Am introns from the parental colonies used in the Coral Bay crosses. However, only the mini-collagen intron seemed to show useful variability for the genotyping, with every parental used in the crosses having a distinct genotype (Figure 5.4). For AFO53, three of the parentals showed the same heterozygote genotype and the remaining colony was a homozygote for the shorter allele present in the heterozygotes. The cnox2Am intron did not show any variation among the parentals.

The fertilization rate in controls using only heterospecific sperm at Coral Bay (CB) was very low: 3.47 % ± 0.94 (average ± standard error). This fertilization rate was much lower than previously obtained values for these two species of c.a. 30% (Mackenzie and Willis, unpublished data) and made the use of these crosses to assess the role of interspecific sperm competition unreliable due to the small number of resulting hybrid embryos. If conspecific sperm had any effect, it would have been impossible to assess it statistically. Therefore I did decide not to follow up the genotyping of the embryos.

Discussion

Microsatellites

The most critical step in the microsatellite development procedure is ligating the adapters to the restriction fragments. Hence, I expected the method to have failed at this step. To examine whether this had really happened, I sequenced three random clones from before probing and each displayed random genomic sequence of the expected size range, although none contained microsatellites. Therefore, a possible explanation for the failure is that the column preferentially selected for concatemers, although not exclusively.

The recovery of microsatellites from acroporid corals has been generally unsuccessful. Five other attempts to develop microsatellites in *Acropora*, representing four distinct methodological approaches undertaken within three independent labs, also failed (Márquez et al., in press). Apart from the technical problems involved in the development of microsatellites, there is now clear

evidence that biological constraints can affect both the abundance and motif composition of microsatellites in the genomes of different taxa (Tóth et al. 2000). For example, the abundance of microsatellites is much lower in the genomes of birds versus mammals (Primmer et al. 1997), as well as plants versus vertebrates (Lagercrantz 1993). Genome specific rarity of microsatellites such as demonstrated in Lepidoptera (Saccheri and Bruford 1993; Nève and Meglécz 2000) and Onycophora (Sunnucks 2000) may explain the difficulties that many researchers have experienced developing microsatellites, despite their use of techniques which were both common and successful in other invertebrates and vertebrates. It is well documented that genome size is directly correlated with microsatellite abundance (Hancock 1996). At the D.J. Miller lab we have estimated the genome size of *Acropora millepora* by quantifying the amount of DNA extracted from known quantities of sperm and from the frequency of single copy genes in gDNA libraries (unpublished data). According to these estimates, the amount of DNA per cell in this coral species is one order of magnitude smaller than that in mammals. Thus, if the small genome size of acroporid corals correlates with low microsatellite abundance, rarity may explain some of the difficulties I have faced with microsatellite development.

Spawning failure

Retrospectively, it is not surprising that the '99 spawning season was marked by failure, given the bad weather conditions and the close association between spawning, temperature and water movement (or lack of it) (Babcock et al. 1986). Moreover, the mass bleaching event of '98 seemed to have had a major detrimental impact on the reproduction of corals in '99, in particular on the *Acropora* spp. (Page and Willis in prep.) and soft corals (Michalek-Wagner and Willis 2001).

Fertilization Patterns

The mixture of self and conspecific sperm in the proportion 100:1 showed reduced fertilization rate in comparison to other proportions and to the conspecific sperm control. As self-fertilized eggs commonly develop abnormally

and disintigrate rapidly (BL Willis and LM Márquez, personal observation), it is possible that this reduction in fertilization reflects higher selfing (in accordance to the higher proportion of self-sperm) followed by mortality of embryos.

The fertilization rates using mixtures of self and heterospecific sperm were equal or smaller than when self-sperm alone was used. Therefore there is no reason to assume that the “mentor effect” is important in these corals. The explanation for the range of self-compatibility observed in corals must lie in molecules related to binding and/or fusion of sperm and eggs, as is the case for cross compatibility. The work to uncover what molecules these might be and how the system may function has just begun (Chapter 6). In ascidians, where the molecular basis for gamete recognition is better understood, it has been demonstrated that selfing and hybrid blocks operate at the level of the vitelline coat but have separate mechanisms (reviewed in Byrd and Lambert 2000). It has been proposed that the "mentor effect" in plants occurs because the recognition system to avoid selfing and hybridization is the same and heterospecific gametes interfere with the system promoting selfing. Therefore the absence of "mentor effect" in my experiments suggests that in corals, as in ascidians, the selfing and hybrid blocks have also separate mechanisms.

Crosses involving conspecific sperm had a higher fertilization rate than the heterospecific control, independent of the proportion of conspecific vs. heterospecific sperm. Although genotyping data are necessary to reach a conclusion, this observation suggests that heterospecific sperm do not reduce the conspecific fertilization rate in these *Acropora* species, as it has been shown to occur in ascidians (Lambert 2000). Alternatively, small amounts of conspecific sperm may facilitate heterospecific fertilization, providing some sort of mentor effect. This could explain the high fertilization rates with only 1% of conspecific sperm. Nevertheless, without the determination of paternity it is impossible to assess whether the number of hybrids is smaller or larger in crosses using the mixture of sperm than in exclusively heterospecific crosses. In conclusion, additional experiments involving paternity analysis and species with higher cross-compatibility are needed to support the argument that conspecific sperm precedence may be the mechanism that maintains highly cross-fertile coral species as independent lineages (Chapter 4).

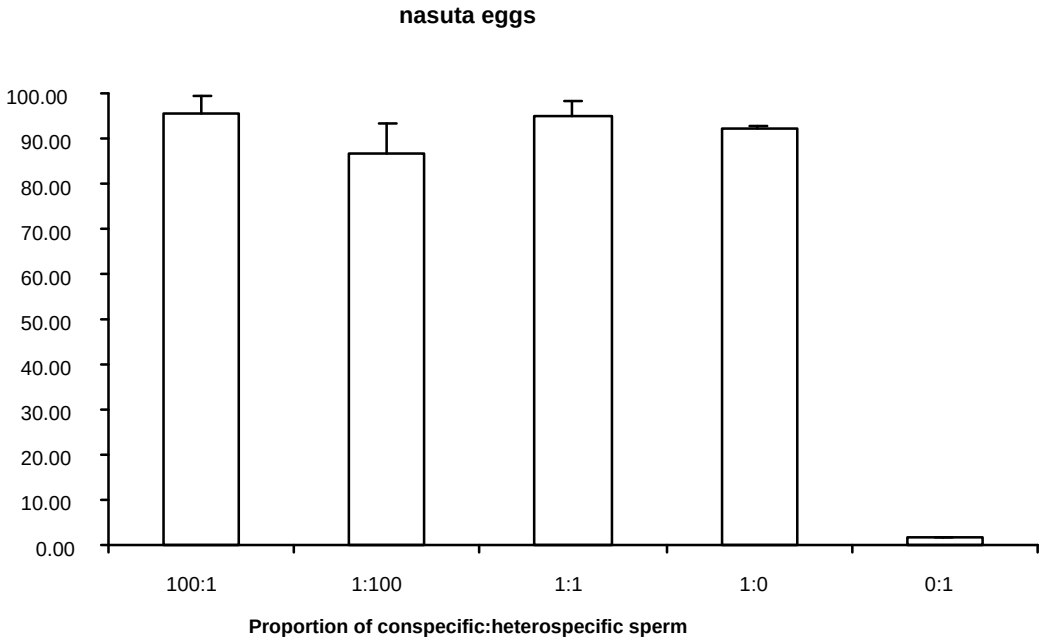


Figure 5.1 Fertilization rate of *Acropora nasuta* eggs using mixtures of conspecific sperm and *A. cerealis* sperm. Two crosses were performed involving two different *A. nasuta* colonies and the same *A. cerealis* colony. Each treatment consisted of aproximately 100 eggs incubated in the mixture of sperm at a concentration of 10^6 sperm/ml in 25 ml glass vials. Each treatment was replicated three times.

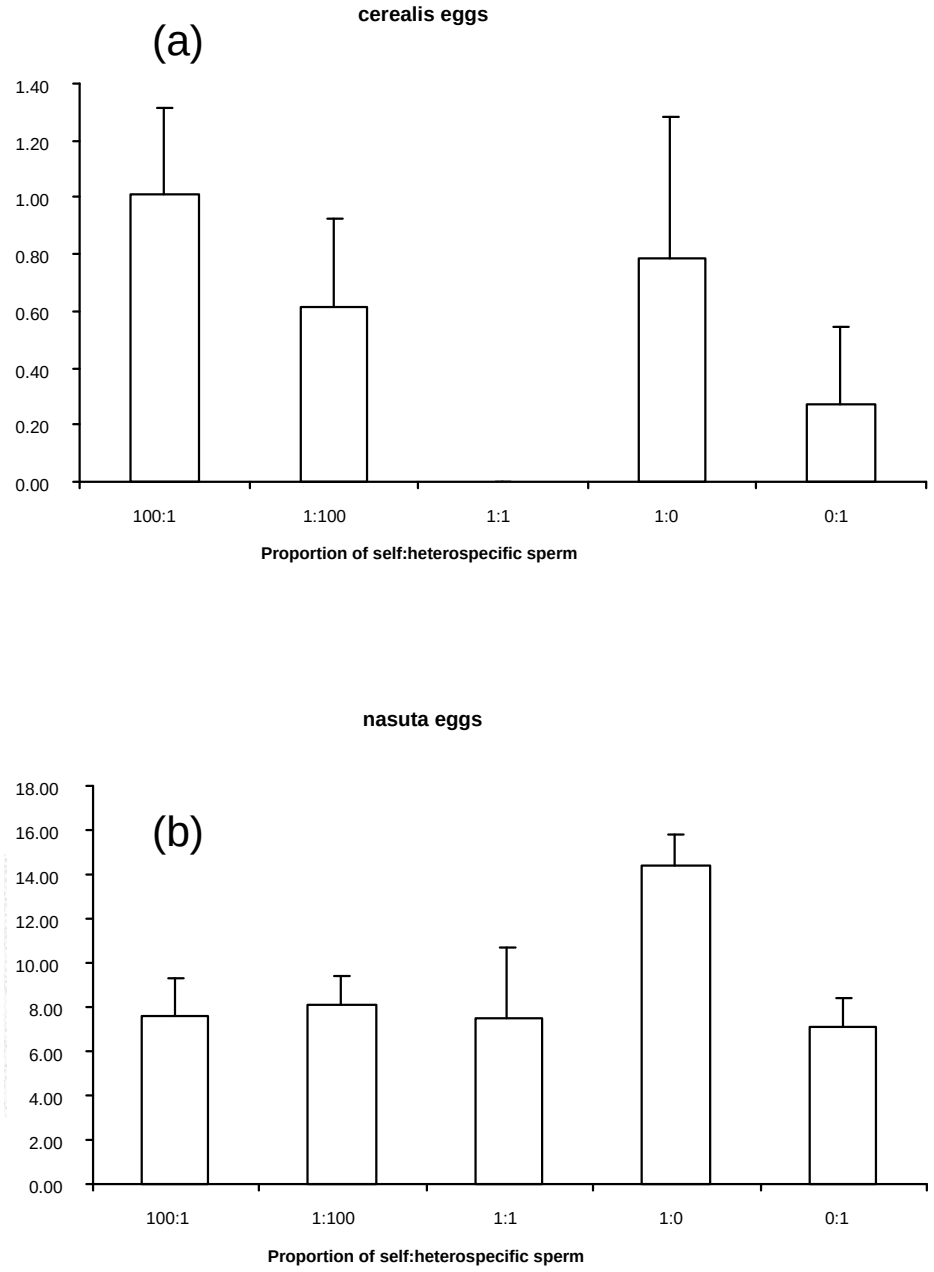


Figure 5.2 Fertilization rate of *Acropora cerealis* eggs using mixtures of self-sperm and *A. nasuta* sperm (a), and of *A. nasuta* eggs using mixtures of self-sperm and *A. cerealis* sperm (b). One cross was performed for each type of eggs. Treatments were designed as explained in Figure 5.1.

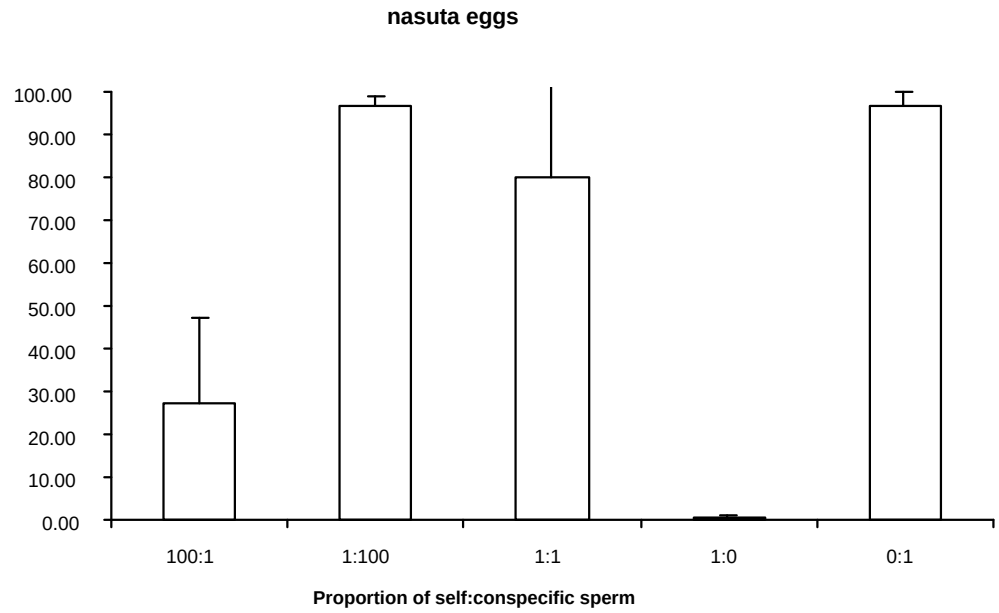


Figure 5.3 Fertilization rate of *Acropora nasuta* eggs using mixtures of self-sperm and conspecific sperm. Two crosses were performed involving two different *A. nasuta* colonies. Treatments were designed as explained in Figure 5.1.

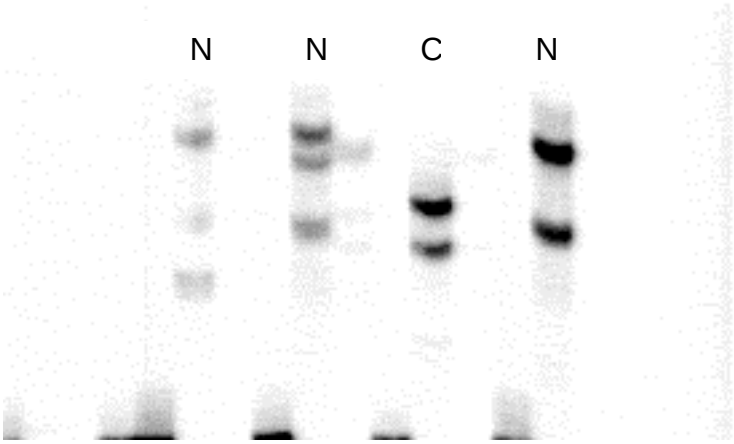


Figure 5.4 Single stranded conformational polymorphisms (SSCP) of Mini-collagen from the parental *Acropora* colonies employed in sperm competition trials. C= *A. cerealis*, N1= *A. nasuta* colony 1, N2= *A. nasuta* colony 2, N3= *A. nasuta* colony 3.

Table 5.1 Treatments employed to test for sperm competition in *Acropora*.

SPERM SOURCE	EGG SOURCE			
	nas 1	*nas* 2	*nas* 3	*cer* 1
None	Control	Control	Control	Control
nas 1: *cer* 1 100:1 1:1 1:100 1:0 0:1	Self vs. Heterospecific + Controls	Conspecific vs. Heterospecific + Controls	Conspecific vs. Heterospecific + Controls	Self vs. Heterospecific + Controls
nas 2: *cer* 1 100:1 1:1 1:100 1:0 0:1	Conspecific vs. Heterospecific + Controls	Self vs. Heterospecific + Controls	Conspecific vs. Heterospecific + Controls	Conspecific vs. Heterospecific + Controls
nas 1: *nas* 2 100:1 1:1 1:100 1:0 0:1	Self vs. Conspecific + Controls	Self vs. Conspecific + Controls	Conspecific vs. Conspecific + Controls	Heterospecific vs. Heterospecific + Controls
nas 1: *nas* 3 100:1 1:1 1:100 1:0 0:1	Self vs. Conspecific + Controls	Conspecific vs. Conspecific + Controls	Self vs. Conspecific + Controls	Heterospecific vs. Heterospecific + Controls
nas 2: *nas* 3 100:1 1:1 1:100 1:0 0:1	Conspecific vs. Conspecific + Controls	Self vs. Conspecific + Controls	Self vs. Conspecific + Controls	Heterospecific vs. Heterospecific + Controls

Table 5.2 List of primers, the length of the amplified regions and their respective melting temperatures for markers used in genotyping of embryos of acroporid corals.

Marker	Primer	Sequence (5'-3')	Length (bp)	Tm (°C)
AFO53		CGCCCAAAGTAGTCGCAAA	~130	50.5°
		GATTCATCTTGGGATAAATGGA		
Mini-collagen	FP1	TGTACTTGCATCGTGTCTTGTAGCCATAG	275	65°
	IRP	TCAAAAAGAAAAGCGAGAGGC		
C-Nox 2	FP1	GCAAGTGCCGTTCATCCTTTTC	440	52°
	RP1	GCGAATTGACGACAACTCTGTTC		

Chapter 6 In search of the molecular basis of hybridization in Acropora: the role of the coral egg integrin b_{Cn1} in fertilization

Abstract

Integrins are proteins involved in cell adhesion that play major roles in gamete binding and fusion in mammals. A cDNA sequence encoding for a β1-class integrin has been identified in the scleractinian coral *Acropora millepora.* Given that the integrin mRNA is present in unfertilized eggs, the corresponding protein may have a potential role in coral fertilization. As a first attempt to elucidate the molecular basis of gamete specificity in corals, I studied the role of the *Acropora millepora* β_{Cn1} integrin in fertilization. I examined the effect of polyclonal antiserum raised against a substantial part of the β_{Cn1} integrin on fertilization rates of *A. millepora* eggs. Fertilization rates were significantly reduced in the presence of the antiserum (68.71% ± 4.16) in relation to both the seawater (87.39% ± 2.38) and the serum controls (83.42% ± 3.19). Phase-contrast microscopy of treated and untreated eggs revealed that the antibodies significantly affected sperm binding. Moreover, the antibodies were also able to inhibit fertilization in the case of eggs of *A. tenuis*, a genetically distinct species from *A. millepora*. Given the incomplete inhibition of fertilization and the lack of apparent specificity of the antibodies (inhibiting fertilization in both *A. millepora* and *A. tenuis*) I conclude that β_{Cn1} Integrin must act in concert with other factors to confer the (limited) specificity of gamete interactions observed in acroporid corals. Nevertheless, my results implicate disintegrin-integrin binding in the fertilization process in *Acropora*, and suggest that some functions of these molecules may have been conserved in corals and humans.

Introduction

The molecular basis of gamete specificity has been studied in relatively few animals. Nevertheless, this literature provides a theoretical background with which to approach the coral system. Abalone and sea urchins are also marine invertebrates with external fertilization. Recent advances in understanding the

specificity of gamete interactions in these systems may therefore appear directly relevant to the study proposed here. However, careful consideration of the literature suggests otherwise; as discussed below, it is extremely unlikely that the determinants of fertilization specificity in corals are related to those described in mollusks and echinoderms. The most highly studied system is that of the mouse, and given the precense in coral eggs of a protein structurally homologous to one in mouse involved in sperm-egg binding (see below), this system is likely to be the most useful comparison as well.

The fertilization process in mammals (reviewed in Flesch and Gadella 2000) involves: 1) the capacitation of sperm cells in the female genital tract; 2) binding of sperm cells to the extracellular matrix of the eggs (zona pellucida, ZP); 3) the acrosome reaction, which is an exocytotic process that makes the enzymatic machinery required for sperm penetration available through the ZP; 4) the meeting of sperm cell meets the plasma membrane of the egg cell (oolemma), after complete penetration; 5) a specific set of molecules in a disintegrin-integrin type anchoring of the two gametes which is completed by fusion of the two gamete plasma membranes; and 6) activation of the fertilized egg and initiation of zygotic development. In mammals, the specificity in sperm-egg interactions seems to occur mainly at the ZP, since fertilization of ZP denuded oocytes by capacitated acrosome-intact sperm leads to polyspermy; furthermore, sperm-oolemma fusion can be achieved with heterologous sperm (Aitken 1994). This is illustrated by the fact that nude hamster oocytes are routinely used to test the fusion competence of human sperm in *in vitro* fertilization (IVF) clinics (Grunfeld 1989).

Strict homologues of the ZP molecules are present only in vertebrates. In the abalone and in sea urchins, proteins that have been considered the functional equivalents of the mammalian ZP molecules have been identified. The elegant work of Vacquier has demonstrated that in the abalone, the sperm protein lysin interacts with the VERL protein on the egg in a species-specific manner (see Vacquier, 1998 and references therein). The (unrelated) sperm protein bindin performs an analogous function in the sea urchin (Gao et al., 1986). Like VERL, bindin contains repeated motifs (however, these are short in bindin but long in VERL), the number and sequence of which vary between closely related species, and which are believed to confer species-specificity (Minor et al.,

1993). Note that, although fulfilling analogous roles and both evolving rapidly through the same molecular mechanisms (i.e. unequal crossing over and concerted evolution; Swanson and Vacquier, 1998), bindin and VERL are unrelated proteins on the opposite sides of the egg-sperm interaction. For these reasons it is unlikely that these specificity-conferring proteins have strict homologues in other animals; rather, VERL and bindin are likely to have evolved independently to address the same requirement in different animal groups.

Coral eggs lack a layer corresponding to the mammalian ZP and the vitelline envelope (which is the outer layer of the egg in echinoderms and mollusks, where the VERL and bindin receptors are located). Furthermore, the characteristics of gamete specificity in corals are completely different to those of sea urchins and abalone; whereas these latter groups show a high level of species specificity, corals do not. The breeding system of *Acropora* shows limited specificity, as interspecific fertilization occurs most readily between species that are morphologically similar (Willis et al. 1997). This leads to the prediction that the specificity of gamete interactions in corals could be based on disintegrin-integrin interactions, i.e. the type that anchor the gametes and fuse the membranes in mammals. The specificity of this system alone could be sufficiently low to permit hybridization with some congeners but not efficient fertilization between distantly related species.

Integrins comprise a large family of cation-dependent heterodimeric trans-membrane receptors composed of non-covalently linked α and β subunits (reviewed in Hynes 1992). Eighteen α and eight β subunits have been identified in mammals, and these subunits form 23 known heterodimers. The ligand specificity for each heterodimer is determined by the specific combination of α and β subunits. Due to the cellular-specific interactions that occur between sperm and eggs, they express a unique repertoire of integrins and molecules that contain integrin recognition sites (reviewed in Bowen and Hunt 2000). In the case of sperm, one of these is fertilin (PH30), a member of a family of proteins called ADAMs (characterized by the expression of a disintegrin and metalloprotease domain). ADAMs are composed of α and β subunits and PH30 is a prime candidate for gamete binding and fusion (Ramarao et al. 1994). The

extracellular domain of the fertilin β subunit posseses a disintegrin “domain” containing the Arg-Gly-Asp (RGD) peptide motif, which is known to function as a ligand in interactions with several integrins (Myles and Primakoff 1997). Sperm express the α5β1 integrin during capacitation and αvβ3 after the acrosome reaction (Fusi et al. 1996). On the other hand, oocytes have been shown to contain integrin subunits α2, α3, α5, α6, β1, β3, β4 and β5 (Evans et al. 1995).

Although the mammalian oocyte expresses many different integrin subunits, the available data highlights the importance of α6β1 integrins on the egg surface, where the complex can facilitate fertilization by interacting with fertilin. Almeida et al. (1995) demonstrated that sperm-egg binding is completely blocked in mice by a monoclonal antibody to the α6 integrin subunit and by a peptide analogous to the integrin ligand domain. However, using an RGD-containing peptide, an antibody to α6, or an antibody against the binding domains of αvβ3 has no effect on sperm-egg binding. Additionally, these same authors have found that spermatozoa bind to somatic cells that express the α6β1 integrin. Moreover, binding to these cells can be inhibited by the fertilin analog. As in the case of mice, RGD-peptides do not block human sperm/oocyte interactions. However, monoclonal antibodies that completely inhibit sperm-egg fusion in the mouse are only partially effective in man (Ji et al. 1998).

The evolutionary history of integrins appears to have been complex. Hughes (2001) published a phylogenetic analysis of vertebrate and invertebrate integrins indicating that two major families of α integrins originated prior to the divergence of deuterostomes and protostomes; analysis of β integrins could not clearly resolve whether β integrin genes duplicated prior to the origin of vertebrates, although it suggested that at least the gene encoding vertebrate β4 may have done so. Previously, Brower et al. (1997) have cloned the entire coding sequence of an *Acropora millepora* integrin, β_{Cn1}. These authors proposed that β_{Cn1} should be considered a representative of the mammalian "β_1-class", based on similarity scores and sequence motifs. However, in Hughes' (2001) phylogeny, β_{Cn1} is basal in a clade composed exclusively of invertebrate integrins. This clade forms a sister group to a clade containing all

the vertebrate integrins. Moreover, neither the phylogeny of α nor that of β integrins showed a close correspondence with patterns of α–β heterodimer formation or other functional characteristics (Hughes 2001). In order to investigate the role of the *Acropora millepora* integrin β_{Cn1} in coral gamete interactions, I carried out experiments based on those described by Almeida et al. (1995), in which the effects of antibodies to the mammalian β1 on sperm-egg binding, fusion and fertilization were examined. Previously, plasmids have been constructed in the Miller laboratory which permit the production of recombinant *Acropora* β_{Cn1} (Hardie 1999). The protein was expressed and purified (by preparative gel electrophoresis) by Dr Julian Catmull, and then used to immunize rabbits, resulting in polyclonal antiserum of high specificity and titre (Miller and Catmull unpublished data). Additionally, I tested whether RGD-containing peptides were able to inhibit fertilization. My results suggest that β_{Cn1} is involved in binding of the sperm and eggs and imply that some functions of integrins in egg/sperm interactions may be conserved in corals and humans.

Materials and Methods

Recombinant protein and antibody production

Integrins contain highly hydrophobic domains that are likely to lead to difficulties in heterologous expression. A recombinant protein corresponding to the N-terminal 155 amino acid residues of integrin β_{Cn1} (which includes the I-domain-like region) was therefore generated by expression of the appropriate PCR fragment from pQE30 (Qiagen). The resulting protein was expressed at high levels, but was recovered in membrane (rather than soluble) fractions and hence could not be purified via affinity chromatography. Therefore the recombinant protein was purified by preparative electrophoresis on SDS-polyacrylamide (15%) gels followed by electroelution. For antibody production, rabbits were immunized using four aliquots of the purified protein (300-400 μg/dose) in Freund's complete (first injection) or incomplete (all other injections) adjuvant. Injections were given at intervals of 2 weeks. One week after the final injection, an aliquot of serum was evaluated for antibody titre by western analysis, and the rabbits bled out within 24 h of this. The blood was allowed to

clot at room temperature for three hours, and stored overnight at 4^{o}C to allow the clot to contract, after which the clot was manually removed and the serum harvested by centrifugation. Kate Hardie, an honours student in the Miller laboratory, carried out the cloning for expression, Dr Julian Catmull expressed the protein, and the antiserum was produced by IMVS Veterinary Services (SA).

Coral collections

Coral colonies were collected from fringing reefs in Geoffrey and Nelly Bays at Magnetic Island (19°15'S, 146°50'E), just off Townsville (Queensland, Australia), and from NE Reef on Orpheus Island (18°31'S, 146°19'E), which is part of the Palm Islands group on the inshore shelf of the Central Great Barrier Reef.

Gamete preparation

Gravid coral colonies were collected the afternoon prior to mass spawning nights and placed into separate plastic containers. Gamete preparation followed Willis et al. (1997).

Fertilization trials

Treatments to test for the inhibition of fertilization by antibodies anti β_{Cn1} integrin in *A. millepora* are described in Table 6.1. A total of nine *A. millepora* colonies were used for the crosses, although not simultaneously but over three spawning seasons (two at Magnetic Is. and one at Orpheus Island) due to the difficulty of coping with the crosses within the limited time frame of four hours during which the gametes retain optimal viability (Willis et al. 1997). The reciprocal of each egg-sperm combination was considered to be a separate cross (after Willis et al. 1997). Two crosses were employed to test the effect of two sperm concentrations, 10^5 and 10^6. Having established tha the optimal sperm concentration was 10^6, ten crosses using that concentration tested the effect of and 0.5 dilution of serum containing antibodies, and one tested the effect of 0.1 dilution of the serum containing antibodies. All crosses were

performed in 1 ml volumes in 3 ml glass vials. Between 10 and 40 eggs were incubated, with the respective mixtures for each treatment, for at least 15 minutes before adding the sperm. Vials were incubated by simply leaving them sitting on a bench at room temperature. Unfertilized eggs and developing embryos were fixed with formalin-β-glycerophosphate 6 hours after adding the sperm, and counted under a dissecting microscope.

To determine whether the antiserum inhibited binding of sperm to eggs, I took phase-contrast microscopic pictures of treated and untreated eggs, which were fixed 45 minutes after adding the sperm. Eggs were washed with filtered seawater to remove unbound sperm prior to slide preparation.

To test whether antibodies against β_{Cn1} integrin of *A. millepora* were specific, I performed one fertilization trial with full sperm concentration and 0.5 serum dilution, using eggs and sperm taken from two colonies of *A. tenuis*. *A. tenuis* is very distinct from *A. millepora* using a wide range of molecular markers (van Oppen et al. 2001a; Chapters 3 and 4). This difference is believed to be due to reproductive isolation (van Oppen et al. 2001a), given that *A. tenuis* spawns 2-3 hours earlier than most *Acropora* species (Babcock et al. 1986).

Data analyses

I used a nested ANOVA with two levels (cross and treatment) to test for the effect of the antibodies inhibiting fertilization among the 10 crosses. The requirements of normality and homogeneity of variances were accomplished after using the arcsine transformation, commonly recommended for percentages (Sokal and Rohlf 1995).

To test for paired differences between treatments in the case of one or two crosses for which normality was not observed given the low number of samples, I used the nonparametric Mann-Whitney U test (Sokal and Rohlf 1995).

RGD-containing peptides

I tested whether the peptide GRGDSP, containing the RGD disintegrin "domain", and the peptide control GRGESP (synthesized at the University of Arizona's peptide synthesis facility) inhibit fertilization in *A. millepora* by

performing a fertilization trial. Each of the peptides was added to separate treatments to obtain a final concentration of 200 μM. As with the antibody treatments, eggs were incubated with the peptides for about 15 minutes before adding the sperm for a final concentration of $\approx 10^6$ per ml.

Results

Anti-integrin antibodies inhibit sperm binding

Phase-contrast microscopy revealed that the antiserum drastically affected sperm binding. In Figure 6.1A and B large amounts of sperm were observed bound to eggs pre-incubated with serum control (not containing antibodies anti-β_{Cn1}). In contrast, none or very few sperm were observed bound to eggs pre-incubated with the antiserum anti-β_{Cn1} (Figure 6.1C and D).

Anti-integrin antibodies inhibit fertilization in A. millepora

Fertilization rates were markedly reduced when *A. millepora* eggs were pre-treated with the antiserum developed against β_{Cn1} integrin (Figure 6.2). In the treated eggs, the fertilization rate was reduced to an average of 68.7%, in relation to 87.4% in the seawater and 83.4% in the serum controls. Although the probability associated with the F value for the difference among treatments was slightly higher than the 0.05 rejection value (Table 6.2), the lower mean for the integrin-treated eggs suggests that the antibodies may have blocked some integrin molecules on the eggs' surface, consequently reducing the binding of sperm, and inhibiting fertilization. The variability among crosses was large and highly significant (Table 6.2), probably affecting the significance of the effect of the treatments when data from crosses were combined

The antibodies against β_{Cn1} Integrin inhibited fertilization in a dose dependent manner (Figure 6.3) (Mann-Whitney, $p<0.01$). The 1:10 dilution of antiserum in seawater did not have a significant effect on the fertilization rate, in comparison to either the seawater or serum control (Mann-Whitney, $p>0.05$). The use of a lower concentration of 10^5 sperm/ml resulted in a higher variability, but in average the amount of fertilization was not reduced significantly in comparison

to crosses using the higher concentration of 10^6 sperm/ml (Figure 6.4) (Mann-Whitney, p=0.26).

The inhibitory effect of anti-integrin antibodies is not species specific

The experiments described above indicate that β_{Cn1} integrin is involved in sperm-egg binding and ultimately in fertilization. To determine whether this protein is also responsible for the limited specificity observed in cross-fertilization trials among *Acropora spp.*, I tested the effect of the antiserum on the fertilization of *A. tenuis* eggs. Although the antibodies were developed against the β_{Cn1} Integrin of *A. millepora*, they were able to inhibit fertilization in crosses involving eggs and sperm of *A. tenuis* (Figure 6.5) (Mann Whitney, $p<0.05$). Curiously, in these crosses the serum control also significantly reduced the amount of fertilization in relation to the sea water control (Mann-Whitney, $p<0.05$).

RGD- and RGE-containing peptides completely blocked fertilization

In two crosses employed to test the effect of the peptides on fertilization, positive controls resulted in 87.57 $\pm$ 12.86 % fertilization rates, but not a single egg was fertilized in either the GRGDSP peptide treatment or the control GRGESP. At this stage it is not clear whether these results are due to real inhibitory effects of the peptides or are artefacts of lab procedures.

Discussion

b_{Cn1} integrin is involved in sperm binding and fertilization in A. millepora

The impact of the antiserum against integrin on fertilization was marginally significant (p=0.051). Nevertheless, considering the large variability in fertilization success among crosses, and the microscopic images showing the inhibition of binding in the treated eggs (Figure 6.1), I conclude that the antibodies against β_{Cn1} integrin of *Acropora millepora* are able to partially inhibit fertilization in this coral species. Breeding trials in corals are characterized by high variability in fertilization rates (Willis et al. 1997) and given that fusion and fertilization are efficient mechanisms that require the binding of just one sperm

(Almeida et al. 1995), it is unlikely that even a high antibody concentration blocks every single integrin molecule. These results indicate that integrin is involved in egg binding and fusion, which is very relevant in biological terms.

b1 integrin subunits are involved in binding and fusion of coral and mammal gametes

Even if the *Acropora millepora* β_{Cn1} integrin is not strictly homologous to the integrin β1-class in mammals, the results of this study indicate that it is likely to play a crucial role in the fertilization process. It has been demonstrated that polyclonal antibodies against β1 integrin in mice moderately inhibited sperm-egg binding, while a monoclonal antibody against the binding domain of β1 Integrin partially inhibited sperm-egg fusion in humans (Ji et al. 1998). The β1 integrin certainly participates in gamete fusion, but it is believed that, as the inhibition of fertilization is not total, it must act in co-operation with multiple integrin/disintegrin couples or other cofactors (Ji et al. 1998). Indeed, one of such cofactors may be the egg surface protein CD9, which may initiate and promote fusion (Miller et al. 2000).

The α6 subunit, on the contrary, is no longer thought to have the primary role that once was attributed to it. The original Almeida et al. (1995) study implied that a monoclonal antibody against the α6 subunit was able to inhibit fusion. However, Evans et al. (1997) demonstrated that the monoclonal antibody against the α6 subunit only inhibits fusion in the case of oocytes that have been ZP-depleted using chymotrypsin. In the case of acid-treated eggs, the antibody does not inhibit fusion, suggesting that somehow the acid treatment is less harsh and does not modify critical membrane proteins. Moreover, the monoclonal antibody against the α6 subunit does not inhibit fertilization of ZP-intact eggs (Evans et al. 1999), despite the fact that antibodies can freely diffuse through the ZP (Miller et al. 2000). It is believed that the chymotrypsin treatment may modify some egg plasma membrane proteins, resulting in a loss of function of some protein(s) and/or a modification of critical protein interactions and make the eggs susceptible to the α6 antibody (Miller et al. 2000). However, as coral eggs lack equivalents of the ZP proteins, it is not clear how directly relevant these mammalian data are to coral gamete interactions.

Specificity of Acropora *sperm-egg interactions may depend on proteins other than* b_{Cn1} *integrin*

The antibodies against the *A. millepora* β_{Cn1} Integrin also inhibited fertilization of *A. tenuis* eggs by conspecific sperm. Given the genetic distinctness and low fertilization success rates *in vitro* between these species, it would have been expected that the antibodies developed using a recombinant protein from *A. millepora* should not have inhibited fertilization in *A. tenuis*. The average cross-fertilization rate between these two species is only about 3%, which is very low in comparison to, for example, the average cross-fertilization rate between *A. millepora* and *A. pulchra* (which has a mean of 45%; Willis et al. 1997). However, given that the experiments using the peptides that are specific for the active site of the integrin did not work, it is premature to strongly conclude that integrin is not involved in the specificity of the reproductive system in *Acropora.* As the antibodies were polyclonal, it may be possible that they recognized and epitopes of the molecule that were common between both species and that are not involved in sperm-egg binding. Nevertheless, the antibodies would block the binding in *A. tenuis* just by obstructing the interaction of the integrin with its counterpart in the sperm.

Results regarding the role of the RGD binding motive are inconclusive

It was surprising that both the GRGDSP peptide and the control GRGESP completely inhibited fertilization. The RGD amino acid sequence is known to be a binding domain for fibronectin (a glycoprotein that contains a functional RGD sequence and is present on human spermatozoa) and can bind to other integrin receptors (see review by Hynes 1992). RGD peptides have been shown to inhibit nematocyst binding to fibronectin in hydra (Ziegler and Stidwill, 1992). RGD peptides have also been shown to inhibit I-cell migration in hydra, and in this case the control RGE peptide had no effect (Zhang and Sarras 1994). RGD peptides may have little or no effect on fertilization, and do not completely block fertilization in any system. In the case of ZP-free mouse eggs, both GRGDS and GRGES did not inhibit either sperm binding or fusion (Almeida et al. 1995). Human gamete fusion was partially inhibited by an RGD-containing peptide

(GRGDTP) (Ji et al. 1998). Addition of a soluble RGD peptide during fertilization of bovine oocytes significantly decreased fertilization as compared to the *in vitro*-fertilized controls, while the addition of non-RGD peptide had no effect on fertilization (Campbell et al. 2000).

Both peptides used in my experiment, GRGDSP and GRGESP, have been previously and successfully used in studies of different types of integrins, serving as an inhibitor and control respectively (e.g. Liang et al. 2000; Chan et al. 2001; Gleeson et al. 2001). Therefore it is unlikely that their specific sequences are the cause of an artifact. Independently of the cause, my results regarding the role of the RGD binding motive are inconclusive due to the inhibition of fertilization in my peptide control.

Conclusions

In conclusion, the results indicate that the fertilization process in *Acropora* involves a disintegrin-integrin interaction, and thus suggests that some functions of these molecules may be conserved between corals and humans. Further research should focus on identifying other membrane proteins associated with the coral β_{Cn1} integrin that may be responsible for the limited specificity observed in the breeding system of *Acropora*.

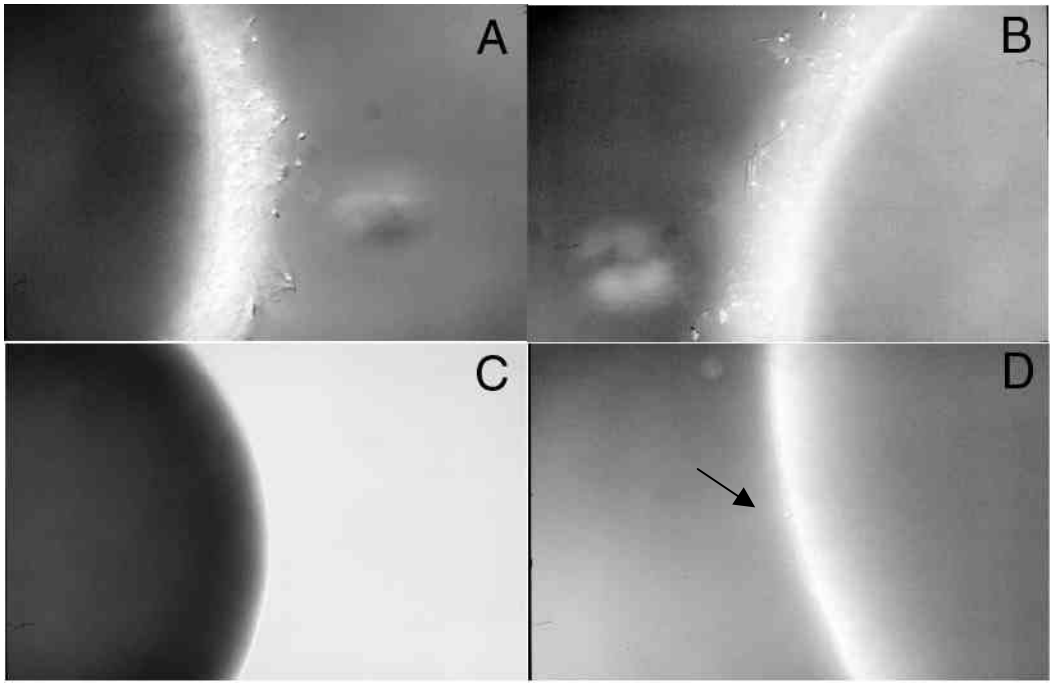


Figure 6.1 Phase-contrast microscopic images of *A. millepora* eggs treated (a, b) and untreated (c, d) with antiserum anti-βcn1 integrin. The arrow indicates the position of a single sperm bound to a treated egg.

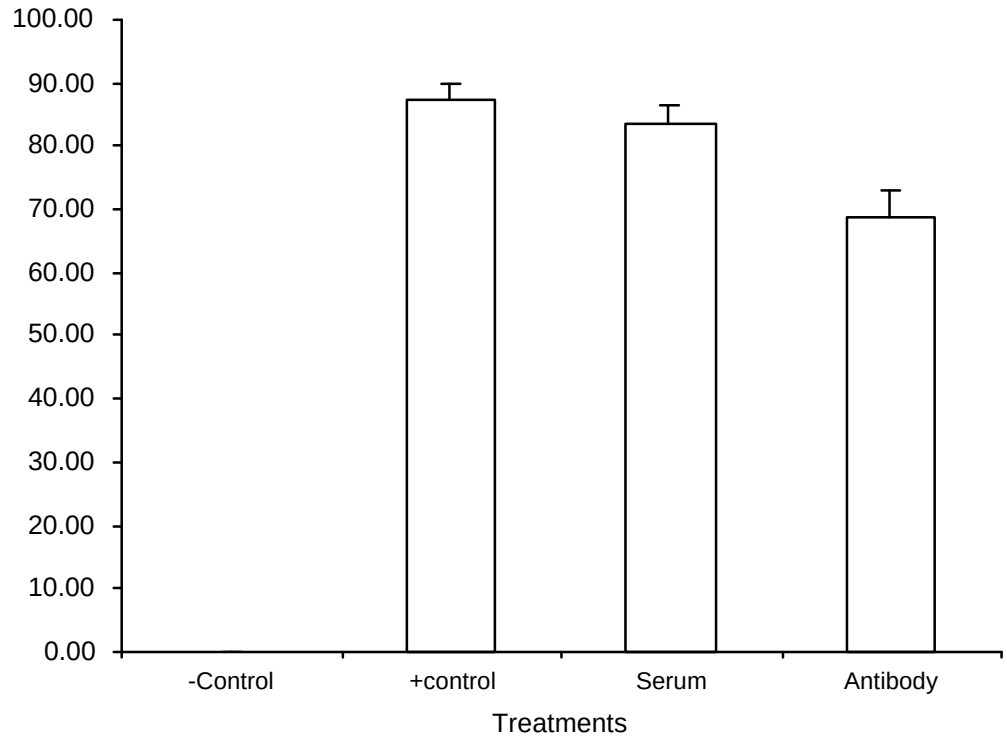


Figure 6.2 Fertilization rates (%) of *Acropora millepora* eggs treated with antiserum anti-β_{Cn1} integrin and controls. Bars show average fertilization rates and standard errors for 11 crosses (n=37). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6/ml. – Control: eggs only added; + Control: eggs and sperm in 1X artificial seawater; Serum: eggs and sperm in 0.5 dilution of rabbit serum in 2X artificial seawater; Antibody: eggs and sperm in 0.5 dilution of rabbit serum containing antibodies against *A. millepora* β integrin in 2X artificial seawater.

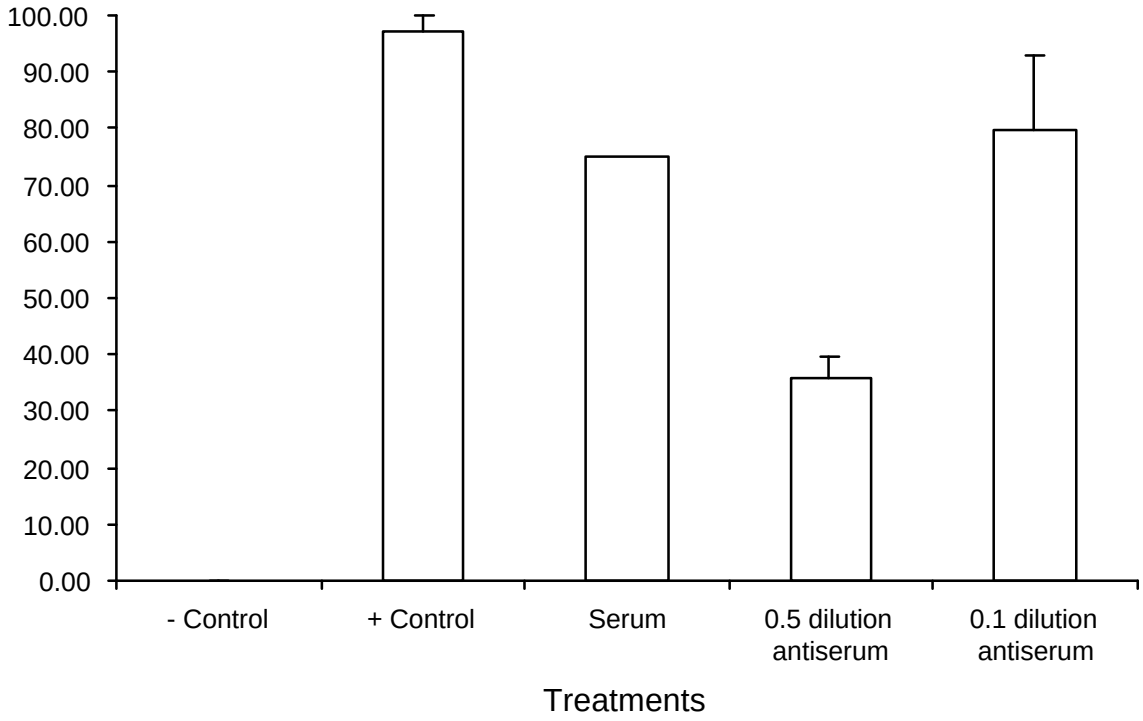


Figure 6.3 Fertilization rates (%) of *Acropora millepora* eggs treated with different doses of antiserum anti-β_{Cn1} integrin. Bars show average fertilization rates and standard errors for a single cross (n=3). Each treatment was repeated three times. Sperm were added at a concentration of 10^6 /ml. Treatment descriptions as in Figure 6.2, with the exception of the dilution of the serum with antibodies

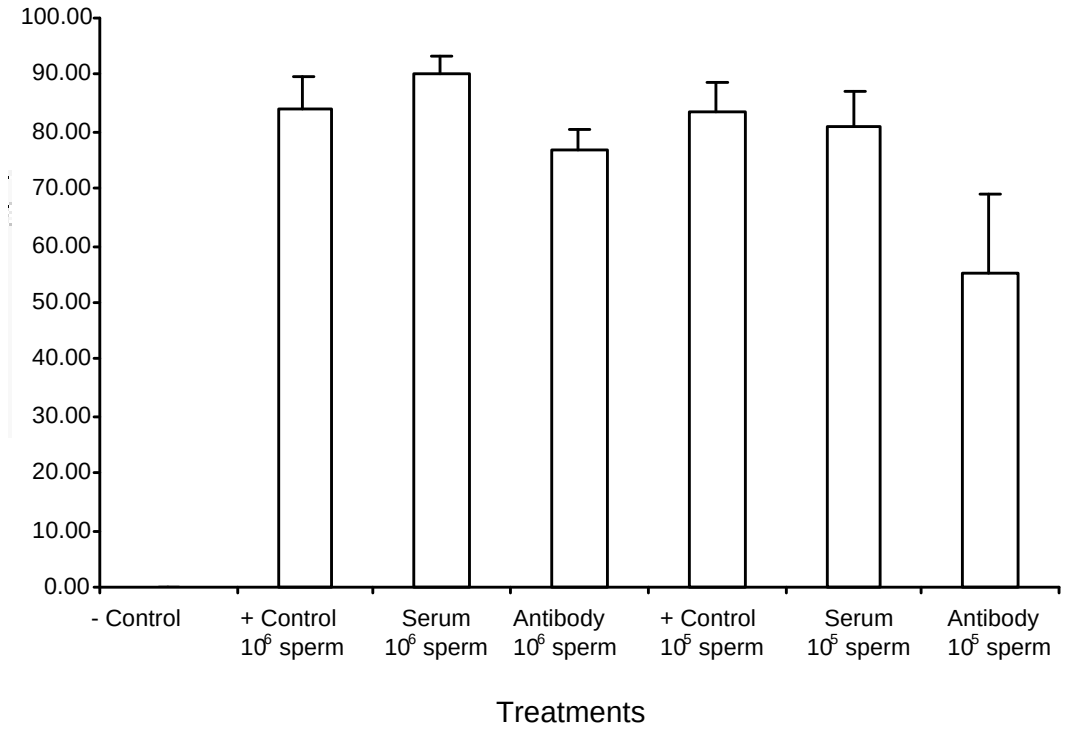


Figure 6.4 Fertilization rates (%) of *Acropora millepora* eggs treated with antiserum anti-β_{Cn1} integrin treatment and controls involving two different sperm concentrations. Bars show average fertilization rates and standard errors for 2 crosses (n=6). Each treatment was repeated three times per cross. Treatment descriptions are as in Figure 6.2, except for sperm concentration that is indicated in the legend for each bar.

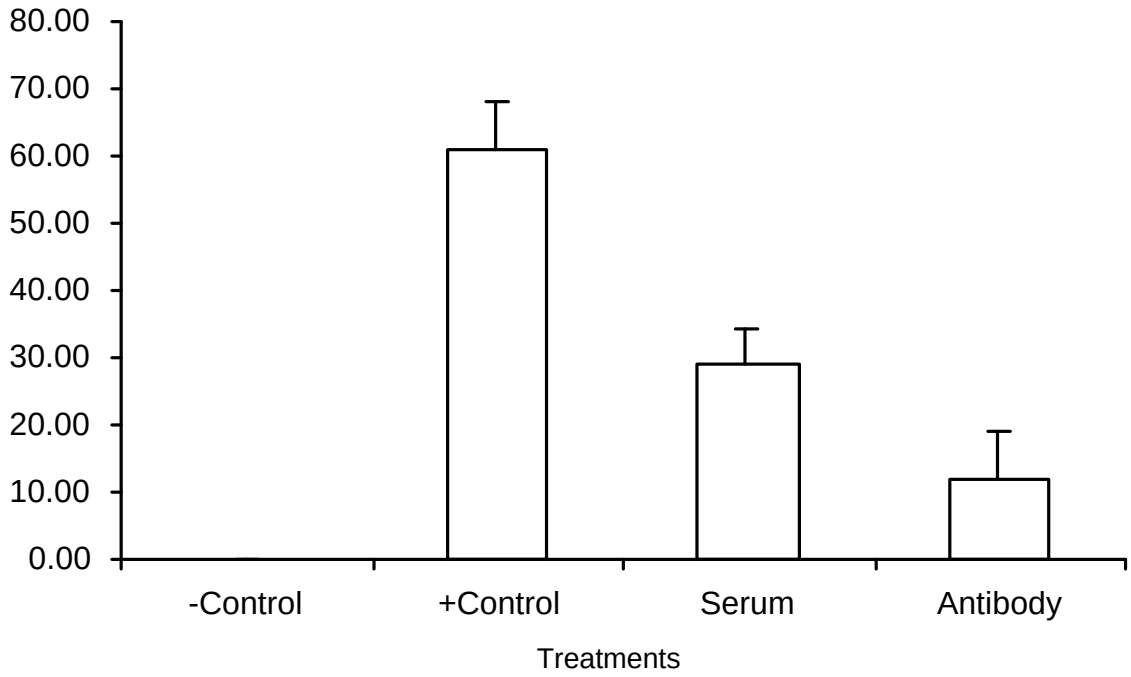


Figure 6.5 Fertilization rates (%) of *Acropora tenuis* eggs under different treatments. Bars show average fertilization rates and standard errors for 2 crosses. Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 /ml. Treatment descriptions are as in Figure 6.2.

Table 6.1 Treatments employed to test fertilization inhibition by antiserum against integrin β_{Cn1} of *Acropora millepora*. Individual crosses were replicated three times for each treatment.

Treatments	Composition 0.5 ml artificial 2 X sea water	0.5 ml distilled water	100µl $\geq 10^6$ sperm	100µl 10^5 sperm	0.5 ml Rabbit serum	0.5 ml Rabbit antiserum
Negative control (eggs only)	√	√				
Positive control Full sperm conc.	√	√	√			
Positive control diluted sperm	√	√		√		
Serum control Full sperm conc.	√		√		√	
Serum control diluted sperm	√			√	√	
Antibody Full sperm conc.	√		√			√
Antibody diluted sperm	√			√		√

Table 6.2 ANOVA table for breeding trials using *Acropora millepora* eggs incubated with antibodies against β_{Cn1} integrin (arcsine transformed data). F'_s= F adjusted for nested design with unequal sample size.

Source of variation	*df*	Sum of Squares	Mean square	F_s or (F'_s)	*P*	Explained variance
Among treatments	2	2.17	1.08	3.320 (3.145)	0.0514 0.0592	20.9 %
Among crosses	27	8.82	0.33	10.21	<0.001	63.3 %
Within crosses	62	1.98	0.03			15.8 %
Total	100	12.97				

Chapter 7 General Conclusions

Introgression, pseudogenes and the phylogenetic utility of rDNA in *Acropora*

Recent studies on oaks have given rise to concern about the use of the rDNA ITS-5.8S region for phylogenetic analysis of hybridizing species due to the presence of highly divergent sequences, some of which are likely to be pseudogenes (Mayol and Rosselló 2001; Muir et al. 2001). Accordingly, one of my objectives was to reassess the phylogenetic utility of the ITS-5.8S region of the rDNA in *Acropora*. Due to extremely high levels of variability and the resulting difficulties in alignment that I found for 18 species of *Acropora*, I do not recommend using ITS sequences for phylogenetic analyses of this genus. However, the 5.8S rRNA gene appears to provide an appropriate level of phylogenetic signal, and the major features of the 5.8S rRNA phylogeny are congruent with those previously obtained using a single copy nuclear intron and a mtDNA intergenic region (van Oppen et al. 2001a).

Results from several independent tests, including: examination of expressed 5.8S types; the occurrence of deamination-like substitutions at methylation sites; differences in evolutionary rates among clades of the 5.8S phylogeny; and ocurrence of non-compensatory mutations that may affect the rRNA secondary structure, suggest that sequences in one of the eight phylogenetic clades represent pseudogenes. The origin of these pseudogenes may lie in hybridization events that brought together divergent rDNA families. As the mismatch repair machinery seems to be sensitive to remote mismatches (Petit et al. 1991), high divergence in the spacer regions of rDNA copies coming from different species may suppress recombination across the entire rDNA array impeding concerted evolution (Muir et al. 2001). Alternatively, hybridization may produce chromosome rearrangements (Riseberg et al. 1995) that can relocate rDNA copies at different chromosomal positions, reducing the homogenizing action of concerted evolution (Arnheim et al 1980). Depending on the amount of time elapsed since the hybridization event, and on whether the rDNA types are silenced or not, they may become pseudogenes. Thus, even pseudogenes may

be valuable for the study of reticulate evolution, signaling past hybridization events.

Future work should focus on sequencing more cDNA clones from several *Acropora* species to determine how many rDNA types are expressed. *In situ* hybridization is also needed to determine whether the divergent rDNA types in *Acropora* are present in more than one nucleolus organizer region (NOR).

Introgression versus ancestral polymorphism in *Acropora*

Distinguishing between introgression and ancestral polymorphisms in phylogenetic analysis can be especially difficult when species are of relatively recent origin and the fossil record is scarce (Avise 1999), as appears to be the case in the genus *Acropora* (Wallace 1999). Nevertheless, coalescence times for acroporid species should be relatively short, because their effective population sizes are predicted to be small (chapter 4), despite very large and highly connected contemporary populations. Short coalescence times, high potential for cross-fertilization (Willis et al. 1997) and the correlation of major clusters in the trees with species that are temporally isolated reproductively (i.e. differ in the timing of spawning) (van Oppen et al. 2001a; Chapters 2 and 4) favor the introgression hypothesis.

As discussed in the introduction, one of the major difficulties in distinguishing between introgression and ancestral polymorphism in corals is the lack of well defined hybrid zones as a consequence of their high dispersal potential. In contrast, even when several terrestrial taxa in Europe have a recent origin, they have well identified hybrid zones that have allowed establishing that patterns of allele or haplotype sharing are due to introgression (reviewed in Hewitt 2000, 2001).

In vitro cross-fertilization trials have been undertaken only among spawning species of corals, due to logistical difficulties associated with obtaining and manipulating gametes of brooders. Studies of the likelihood of hybridization among brooding species, which have philopatric larvae and more genetically subdivided and distinctive distributions than spawning species (Ayre and Hughes (2000), may lead to the identification of hybrid zones in corals. If hybrid zones are located, then there will be a greater chance of differentiating between

introgression and ancestral polymorphism as alternative explanations for the low genetic differentiation found among many species of *Acropora*.

Why do highly cross-fertile corals remain distinct?

Despite high cross-fertility *in vitro*, natural populations of *A. cytherea* and *A. hyacinthus* are genetically distinct and the two morphospecies constitute different evolutionary lineages. Nevertheless, natural hybridization is likely to occur between these two species, albeit at much lower frequencies than those implied by *in vitro* cross-fertilization trials, because there are no fixed allele differences, genetic distances are small and phylogenetic analyses suggests that they form part of a large syngameon.

Why does hybridization appear to occur less frequently, as indicated by the genetic data, than would be predicted based on *in vitro* breeding data? One possibility is that cross-fertilization trials simply overestimate rates of hybridization occurring under natural conditions, because the do not consider factors such as sperm competition. Unfortunately, my experiments designed to test the effect of sperm competition on rates of hybridization were unsuccessful because of spawning failure and low cross-fertilization between colonies of the study species. Further experiments using colonies with higher cross-compatibility and SSCP of single copy genes (such as mini-collagen) for the determination of paternity, are needed to support the hypothesis that conspecific sperm precedence may be the mechanism that maintains highly cross-fertile coral species as independent lineages.

Alternatively, negative selection on hybrids, disruptive selection on the parentals and/or isolation of hybrids from parentals may maintain cross-fertile species as genetically distinct entities. Hybrid colonies have been maintained under laboratory conditions for up to three years, but it is not yet clear whether they are capable of reproducing (B. Willis, unpublished data). Preliminary observations indicate that survival rates of hybrids under field conditions are similar to those of purebreds (B. Willis unpublished data). Further work on these two subjects is needed to assess the fitness of coral hybrids. Additionally, quantification of ecological differences between cross-fertile species would help to address the significance of disruptive selection. A third promising line of

research is the study of chromosomal rearrangements, which have been used to explain the isolation of hybrid species from parentals in sunflowers (Rieseberg et al. 1995; Rieseberg 2000), and may be relevant to the formation of species such as *A. prolifera* in the Caribbean.

Future of coral systematics

Comparison of the results of my study on the *Acropora hyacinthus* group with those of other species groups in the genus supports the argument of Wallace and Willis (1994) that the taxonomic status of each species has to be reassessed on a case-by-case basis. In the Caribbean, hybridization between *A. palmata* and *A. cervicornis* has produced a new species, *A. prolifera* (van Oppen et al. 2000). This thesis demonstrates that *A. hyacinthus* and *A. cytherea* remain distinct, despite small genetic distances and high cross-compatibility. Ribosomal DNA data (van Oppen et al. submitted) and preliminary allozyme results (Willis and Ayre unpublished) suggest that *A. millepora* and *A. pulchra*, another pair of species with high cross-fertility rates (Willis et al. 1997) are genetically indistinguishable. However, additional Nested Clade Analysis of single copy nuclear markers would be informative with respect to the effect and extent of hybridization between *A. millepora* and *A. pulchra*.

All of these discoveries in molecular systematics have implications for taxonomy. However, changes in taxonomy involve a great deal of responsibility, because they affect other disciplines such as ecology, palaeontology, biogeography and conservation (Knowlton and Weigt 1997; Knowlton 2001). Therefore, for the time being, and particularly in the case of *Acropora*, I advocate taxonomic conservatism and the use of morphological species as operational taxonomic units (Veron 1995) following the latest revision of the genus (Wallace 1999).

The future of coral taxonomy and systematics looks brighter, albeit no less complex, thanks to the incorporation of molecular tools. To better assess the extent of hybridization and the importance of reticulate evolution in scleractinian corals, we need to develop new genetic markers and to apply these to a broader range of species, including representatives not just of the genus *Acropora*, but from other genera and families. Results of the Nested Clade

Analysis on a nuclear intron presented here (Chapter 4) and the successful development of microsatellites for *Seriatopora* (Maier et al. 2001) and *Platygyra* (K.J. Miller personal communication), as well as single copy nuclear introns for *Porites* (van Oppen personal communication) encourage further research on molecular ecology and systematics of corals.

Towards a molecular understanding of coral fertilization and hybridization

To contribute to the elucidation of the molecular basis of the limited gamete specificity in corals, the role in fertilization of the *Acropora millepora* β_{Cn1} integrin was assessed. The results indicate that β_{Cn1} integrin must act in concert with other factors to confer gamete specificity, given the incomplete inhibition of fertilization and the lack of apparent specificity of the antibodies anti-integrin (inhibiting fertilization in both *A. millepora* and *A. tenuis*). Further research should focus on identifying other membrane proteins associated with the coral β_{Cn1} Integrin, such as the homologue of CD9.

The results in Chapter 6 implicate that disintegrin-integrin interactions play a role in the fertilization process in *Acropora*, and suggest that some functions of these molecules may have been conserved between corals and humans. The *Acropora* system has already provided insights into ancestral linkages of homeobox genes and the evolution of the *Pax* genes, and will probably provide further new perspectives on the age, role and evolution of many gene families (Miller and Ball 2000).

Final Summary

This thesis contributes to the understanding of the mechanisms and evolutionary consequences of hybridization in the coral genus *Acropora* providing four main conclusions:

- Part of rDNA diversity in *Acropora* is due to pseudogenes.
- Highly cross-fertile acroporid corals are not morphs within a species.
- Highly cross-fertile acroporid corals conform cohesion species.
- *Acropora* β_{Cn1} integrin is involved in sperm-egg binding but does not confer reproductive specificity.

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