Corals through the light : phylogenetics, functional diversity and adaptive strategies of coral-symbiont associations over a large depth range

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Chapter 6

Semi-permeable species boundaries in the coral genus *Madracis*: The role of introgression in a brooding coral system

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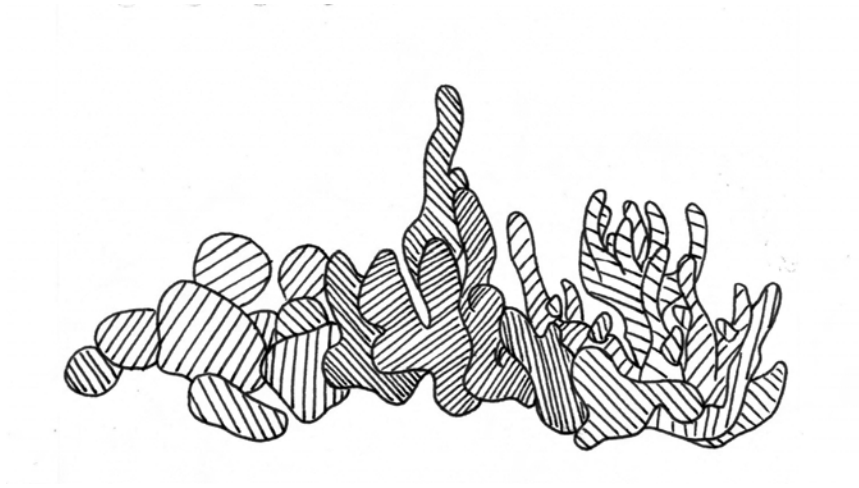

Abstract

Introgressive hybridization and reticulate evolution are described in several phylogenetic studies of mass-spawning corals. However, the prevalence of these processes among brooding coral species is unclear. We used a mitochondrial (*nad5*) and two nuclear (*ATPSα* and *SRP54*) intron markers to explore species barriers in the coral genus *Madracis* and address the role of hybridization in brooding systems. Specimens of six *Madracis* morphospecies of the Caribbean Sea were collected from 5-60 m depth at Buoy One, Curaçao. Polymerase chain reaction–denaturing gradient gel electrophoresis were coupled to detect distinct nuclear alleles within single individuals. The recurrent nDNA phylogenetic polyphyly among taxa is only challenged by *Madracis senaria*, the single monophyletic species within the genus. However, nDNA AMOVAs indicated statistical divergence (0.1% significance level) among morphospecies. mtDNA sequences clustered in two main groups representing shallow and deep water species. *Madracis pharensis* was an exception, with shallow (≤ *c*. 23 m) and deep colonies clustering in different branches, together with their depth-sympatric congenerics. This divergence is repeated for the nDNA suggesting distinct *M. pharensis* depth populations and the hypothetical presence of further unknown genetic pools in deeper reefs. This matched the vertical distribution of the dinoflagellate symbionts hosted by *M. pharensis*, with rDNA ITS2 type B7 in the shallows but type B15 in the deep habitats, suggesting symbiont-related disruptive selection. Non-monophyly of nearly all *Madracis*, the high levels of shared polymorphism and an old fossil record suggest that hybridization is likely to have played a role in the evolution of *Madracis*. Different reproductive traits and symbiont signatures of taxa forming distinct genetic clusters also point to the same conclusion. Furthermore, deep water *M. pharensis* were genetically closer to, e.g., *Madracis carmabi*, than to shallow water conspecifics, evidence for spatially-mediated introgression. Thermoclines may function as a recurrent environmental barrier mediating the aforementioned depth-related processes. We suggest that *Madracis* morphospecies remain recognizable either because introgressive hybridization is non-pervasive and/or because disruptive selection is in action.

Introduction

Scleractinian corals, the key tropical reef-building organisms, have high dispersal potential, large population sizes, ample distributions, overlapping generations and high fecundities (Hughes *et al.* 1992; Palumbi 1994). These characteristics, enhanced by the absence of temporal barriers to interspecific breeding given by synchronised spawning events, provide a great opportunity for interspecific hybridization and introgression, the interspecific exchange of genes that follows. Natural hybridization may cause coral taxa to merge through homogenization of gene pools or may create new hybrid species. Thus, hybridization and reticulate pathways are presently accepted as having played an important role in the evolution of reef corals (Veron 1995; Willis *et al.* 2006). Breeding trials on the Caribbean genus *Montastraea* (Szmant *et al.* 1997) and the Indo-Pacific genera *Acropora* (Willis *et al.* 1997; Hatta *et al.* 1999; van Oppen *et al.* 2002) and *Platygyra* (Miller and Babcock 1997) confirmed that some mass-spawning corals are capable of hybridizing *in vitro*. Moreover, high rates of cross-fertilization between Indo-Pacific species of the mass-spawning genus *Acropora*, suggest that hybridization may have contributed to the high diversity of this genus (Willis *et al.* 2006).

Several molecular studies have also contributed to clarifying such potentially hybridizing systems. The extensive literature on the molecular relationships and evolutionary history of the Indo-Pacific genus *Acropora* (Odorico and Miller 1997; Hatta *et al.* 1999; van Oppen *et al.* 2000; van Oppen *et al.* 2001b; Marquez *et al.* 2002a; Marquez *et al.* 2002b; van Oppen *et al.* 2002; Vollmer and Palumbi 2002) has illustrated that although nominal *Acropora* species constitute genetically distinct entities, they are likely to exchange genes with congeneric species through introgressive hybridization. Even when species are polyphyletic they may constitute statistically distinguishable lineages (Marquez *et al.* 2002b). For many *Acropora* species, the morphology has little predictive value with regard to genotype and this detachment is likely to occur via hybridization. Nevertheless, it is not always possible to distinguish introgressive hybridization from shared ancestral polymorphisms due to incomplete lineage sorting or even from morphological convergence, especially for species that do not show a reliably long fossil record (van Oppen *et al.* 2001b). Another problem for the identification of hybridization cases is that potential hybrids are easily masked by intraspecific variation. Because scleractinian coral morphology is affected by intraspecific variation, caused by genetic differentiation and morphological plasticity as response to the environment (Todd 2008), it is difficult to locate species borders within the morphological continuum.

To date, the only known scleractinian coral for which there is unambiguous genetic evidence for a hybrid origin is the Caribbean species *Acropora prolifera* (van Oppen *et al.* 2000; Vollmer and Palumbi 2002). All colonies of *A. prolifera* were found to be heterozygous for a nuclear intron locus, while allele frequencies were significantly different between sympatric colonies of the putative parental species, *Acropora palmata* and *Acropora cervicornis* (van Oppen *et al.* 2000). Ribosomal DNA ITS sequence types were also shared among all three species (Vollmer and Palumbi 2004). Two additional nuclear loci confirmed the heterozygous character of *A. prolifera*, suggesting that these colonies are most likely first generation hybrids (Vollmer and Palumbi 2002). Finally, mitochondrial DNA established that hybridization occured in both directions and that backcrossing occured with only one of the parental species, *A. cervicornis* (Vollmer and Palumbi 2002). In addition, unlike its two putative parent species, *A. prolifera* lacks a fossil record (Budd *et al.* 1994), is rarer and inhabits more marginal habitats. This sort of

habitat differences, as well as studies on the growth and survival of juvenile hybrids (reviewed in Willis *et al.* 2006), suggest that hybridization may have a role in range expansion and adaptation to changing environments.

The Caribbean *Montastraea* species complex (Knowlton *et al.* 1997; Levitan *et al.* 2004) provides an example supporting the idea according to which hybridization patterns may vary throughout the geographical range of a species (Veron 1995). Both genetic differences and corallite morphological patterns between the three *Montastraea* species were found to be weaker in the peripheral Bahamas and Curaçao than in Panama (van Veghel and Bak 1993; Fukami *et al.* 2004a), suggesting a gradient with the strongest introgression at the geographical extremities of the species distributions. Morphological analyses of fossil specimens offered a large geological time-scale confirmation of this pattern (Budd and Pandolfi 2004), suggesting that semi-permeable barriers at the extreme of species' ranges increase the chance for interspecific hybridization.

Although hybridization events are believed to be widespread on mass-spawning corals, it is not yet clear whether they have the same prevalence among brooding coral species (Willis *et al.* 2006). Brooding corals have internal fertilization and therefore lack the vast opportunity for interspecific breeding known to occur in broadcast-spawning systems. In addition, there is a lack of data on their reproductive periods and their potential for sperm dispersal, and comprehensive cross-fertilization trials are virtually impossible to perform. However, molecular studies of Caribbean species in the genus *Madracis* presented provisional evidence that hybridization may also occur among brooding corals (Diekmann *et al.* 2001).

The Caribbean brooding coral *Madracis* Milne Edwards *and* Haime 1849 is characterised by high levels of morphological plasticity (Bruno and Edmunds 1997) among its six currently recognised zooxanthellate species (Wells 1973a,b; Vermeij *et al.* 2003a). *Madracis pharensis* (Heller 1968) and *Madracis senaria* (Wells 1973a) are encrusting or submassive depth generalist species (5 to > 60 m depth) differing in the number of primary septa (10 and 6, respectively). *Madracis decactis* (Lyman 1859) is nodular, has 10 primary septa, and occurs from 5 to 40 m. *Madracis decactis* overlaps morphologically with *M. pharensis* and their species status is under debate (Fenner 1993; Vermeij *et al.* 2007a). *Madracis mirabilis* Duchassaing *and* Michelotti 1861, *Madracis carmabi* (Vermeij *et al.* 2003a) and *Madracis formosa* (Wells 1973a) are branching species with 10, 10 and 8 primary septa, and are restricted to shallow (2 to 25 m), mid (20 to 40 m) and deep water (> 30 m), respectively. *Madracis carmabi* is suggested to represent a hybrid combining characteristics of *M. decactis* (10 primary septa) and *M. formosa* (branching morphology). Determining the affinity of *M. carmabi* to the other species in the genus will help establishing its taxonomic status.

Diekmann *et al.* (2001) found small and constant genetic variation (approximately 6%) between and within *Madracis* putative species at the ribosomal DNA Internal Transcribed Spacers (rDNA ITS) level. These molecular phylogenies showed that the morphological species distinction only corresponds to monophyletic groups for two of the species: *M. senaria* (with great statistical support) and *M. mirabilis* (less support), while all the other species share rDNA ITS sequences. Two main alternatives have been considered as explanation for the occurrence of this paraphyletic group: recent speciation with incomplete lineage sorting or gene flow through interspecific introgressive hybridization (Diekmann *et al.* 2001).

Going back to cretaceous origin, the *Madracis* fossil record provides arguments for this discussion. For two of the species, *M. decactis* and *M. mirabilis*, the oldest known fossils from

the Caribbean are 15-11 million years old (Budd *et al.* 1994; Budd *et al.* 1995); *Madracis pharensis* appears to be more recent, the only one record being dated about 1.5 million years (Budd and Johnson 1999); and no records are known for *M. formosa*, *M. carmabi* or *M. senaria*. Since the approximate age of the species and the observed sequence divergence within the genus are expected to be reflected in homogenisation of the ITS repeats, the non-monophyly suggests that group of species exchanges genes through introgressive hybridization (Diekmann *et al.* 2001).

As a multigene family, rDNA and the variation among its repeats may not always have been homogenised by concerted evolution. Recent literature shows that rDNA performs poorly for species- and population-level inference in reef building corals (Vollmer and Palumbi 2004). Therefore, we decided to readdress the phylogeny of the genus *Madracis* using multilocus single copy genetic markers from mitochondrial and nuclear genes, as suggested by other studies (van Oppen *et al.* 2001b; Fukami *et al.* 2004b; van Oppen *et al.* 2004). Mitochondrial genomes usually show clonal transmission and are maternally inherited, presenting insight on ancestral sources. Contrasting phylogenies between mitochondrial and nuclear DNA markers may allow the identification of hybridization cases (van Oppen *et al.* 2001b).

Introgressive hybridization is an evolutionary process that can potentially provide raw material for evolution (Anderson and Stebbins 1954) and contributes to colonization of marginal and new habitats (Vollmer and Palumbi 2002). Consequently, unravelling introgressive hybridization and the extent of its occurrence is of high importance today when coral reef ecosystems face serious threats to their biodiversity and resilience (Hoegh-Guldberg *et al.* 2007). Here, we use a mitochondrial and two nuclear intron markers to explore the evolutionary relationships and species barriers in the coral genus *Madracis*, and address the role of hybridization in brooding coral systems.

Materials and Methods

Sample collection

Small fragments (4-16 cm^2) of coral skeleton with living tissue of *Madracis* specimens were collected by scuba diving on the reef slope of CARMABI Buoy One, Curaçao (Figure 6.1), from July to September 2005 and 2006. Chosen colonies correspond to typical morphologies as described above, from depths that represent the distribution of the species. Sampled *M. decactis* and *M. pharensis* colonies were, respectively, clearly nodular and clearly encrusting. *Madracis pharensis* colonies included the three colour morphs in the species: brown, purple and green (Vermeij *et al.* 2002; Frade *et al.* 2008a). Samples were kept in seawater in individual sealed bags and transported to the nearby laboratory of the CARMABI Foundation, where coral tissue was scraped-off and preserved in 95% ethanol and -20ºC until further use. A few *Madracis* specimens originated from other locations (Figure 6.1) were included in the study.

The sample set of 121 *Madracis* colonies comprises 78 colonies from Curaçao CARMABI Buoy One (all six species), 12 from Curaçao Playa Kalki (*M. pharensis* and *M. senaria*), 17 from Aruba (all six species except *M. formosa*), 8 from Trinidad and Tobago (only *M. mirabilis*) and 6 from Bermuda (*M. mirabilis*, *M. decactis*, *M. senaria* and *M. formosa*). All samples were collected at reef slopes between 5 and 60 m. *Madracis myriaster* is the only exclusively azooxanthellate species in the *Madracis* genus and it inhabits the deep Caribbean reefs (Santodomingo *et al.* 2007). One *M. myriaster* sample collected at a depth of 300 m off Buoy One was also included

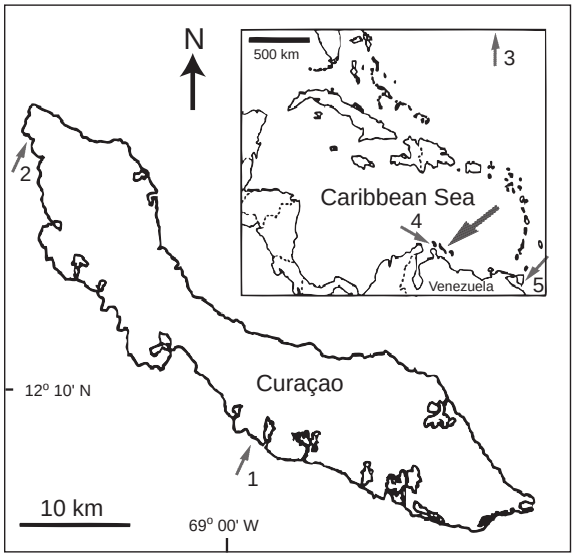

Figure 6.1 The Caribbean Sea (inset), Curaçao (large arrow) and the collection locations: 1, CARMABI Buoy One (Curaçao); 2, Playa Kalki (Curaçao); 3, Bermuda; 4, Aruba, 5, Trinidad & Tobago.

in the study. One non-congeneric species (*Siderastrea siderea*) was sampled to be included as outgroup in phylogenetic analyses. Because samples originating elsewhere than Buoy One were received at a later stage, these were only analysed for the mitochondrial marker. For sample details on species, collection location and depth *see* Table 6.1.

Laboratory procedures

Genomic DNA was extracted using the UltraClean Soil DNA kit (MoBio) according to manufacturer's instructions. Sequence variation was assessed for non-coding exon primed intron-crossing (EPIC) markers previously developed for three different genes: the mitochondrial subunit 5 of NADH ubiquinone oxidoreductase (*nad5*) (Concepcion *et al.* 2006) and the nuclear ATP Synthetase Subunit α (*ATPSα*) and Signal Recognition Particle 54-kDa subunit (*SRP54*) (Jarman *et al.* 2002). A third nuclear intron, ATP Synthetase Subunit *β* (*ATPSβ*), was also included in preliminary surveys but due to the complete absence of sequence variation, this marker was discarded.

The *nad5* intron was successfully amplified using the ND51b degenerate primer pair (NAD5_700F: 5′-YTG CCG GAT GCY ATG GAG-3′; NAD1_445R: 5′ARC CCA ATC GAA ACY TCA TAA CT-3′) of Concepcion *et al.* (2006), reported to amplify a product with an *c.* length of 750 bp.

Since the degenerate primer sets previously available for the two nuclear EPIC markers (Jarman *et al.* 2002) produced non-optimal survey PCR amplifications, alternative nested exon-located conserved regions were identified among a preliminary sequence alignment including all species in the genus *Madracis*. Exon/intron boundaries were identified by comparison with sequences retrieved from GenBank. New exon located primers were designed (ATPSαMadfor2: 5′-ACG AGA ACT TAT CAT TGG AGA CAG-3′; ATPSαMadrev: 5′-GGT GTC AAT CGC AAT AGC TG-3′; and SRP54Madfor: 5′-GAT AAA GTC AAT GAA CTG AAG C-3′; SRP54Madrev2: 5′-TGG AAT TGT TCA TAC ATG TCT C-3′; located, respectively, 54, 14, 11 and 2 bp closer

to the exon/intron boundary).

Because denaturing gradient gel electrophoresis (DGGE) was applied to resolve nuclear gene zygosity (this step was skipped for the cytoplasmatic marker), forward primers were further modified with a 40-bp guanine and cytosine (G-C) rich clamp extension on their 5′-end (Sheffield *et al.* 1989) and renamed (ATPSαMadfor2clamp: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC GAG AAC TTA TCA TTG GAG ACA G-3′; and SRP54Madforclamp: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGA TAA AGT ACC TGA ACT GAA GC-3′).

PCR protocols were optimised for each primer set and used for subsequent amplification of the whole sample set. PCR reactions of 50 μL total volume included 1.0 μL genomic DNA of various concentrations, 1.5 m*M* $MgCl_2$ (in *nad5* increased to 2.125 mM), 200 μM of each dNTP, 125 μM of each primer and 2.5 U of Biotherm Plus Taq polymerase (for the *SRP54* marker, Genscript Taq was used).

PCR cycling conditions included an initial denaturation of 2 min at 94ºC, 37-40 cycles of 94ºC for 20 s, annealing for 30 s, and extension at 72ºC for 60 s. The reaction was completed with 6 min at 72ºC. Annealing temperatures were 51.5, 54 and 48ºC for *nad5, ATPSα* and *SRP54*, respectively. In the case of the nuclear markers, the first 10 cycles were replaced by a "touchdown" procedure (Don *et al.* 1991), to ensure PCR specificity. Here, initial annealing conditions began 5ºC above final temperature and were reduced by 0.5ºC after every cycle. The remaining 30 cycles took place using the respective final annealing temperature.

ATPSα and *SRP54* amplified products (100 ng) were run for 5 h at 100 V and a constant temperature of 60ºC on Bio-Rad DCode DGGE systems under a 20-60% denaturing gradient of urea and formamide. Depending on sequence length, nucleotide composition and sequence variation, the electrophoresis produces differential denaturation and migration of DNA fragments. The G-C clamp stabilizes the melting characteristics of the DNA fragment and ensures that it will remain partially double-stranded under denaturing conditions (Myers *et al.* 1985a; Myers *et al.* 1985b). After electrophoresis and 30 min of Sybergold (Molecular Probes) staining, the gels were photographed over a transilluminator. DGGE profiles were characterised either by a single band (homozygote genotypes) or by quartet banding (heterozygote genotypes). In the last case, two of these bands corresponded to heteroduplexes, the result of re-annealing of heterogeneous DNA single strands during PCR (Buckler *et al.* 1997). All bands were excised, re-amplified and new PCR products loaded on DGGEs to evaluate band isolation. This procedure was repeated once again if needed, to ensure single DNA sequences were obtained. Annealing temperatures were raised 2ºC for all re-amplifications and the "touchdown" procedure was excluded. Re-amplified heteroduplex bands produced their paired homoduplex profiles on new DGGEs. When a band was considered "pure" then the final re-amplification reaction took place using the "non-clamped" primer set and proceeded to sequencing.

All PCR products to be sequenced were purified using the QuickClean 5*M* PCR Purification kit (Genscript). Sequencing was performed with respective forward and reverse primers by Macrogen Korea. Obtained chromatograms were visually inspected and sequences edited and manually aligned in BIOEDIT 7.0.5.3 (Hall 1999).

Additionally, dominant algal symbionts were genetically typed for all *Madracis* specimens using rDNA ITS2 (for methods *see* Frade *et al.* 2008c).

Phylogenetic inference

Polymorphism statistics and uncorrected p-distances (pairwise deletion) were conducted using MEGA 4 (Tamura *et al.* 2007). Standard error estimates were based on 1000 bootstrap pseudoreplicates. Neutrality was tested by Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) using ARLEQUIN 3.1 (Excoffier *et al.* 2005). These tests compare, respectively, the number of segregating sites or the number of alleles (or haplotypes) against their neutral expectation based on the mean number of pairwise differences under an infinite sites model. In order to address potential confounding effects of nuclear loci recombination in the phylogenetic inference, recombination events were estimated by all methods implemented in the program RDP 3.27 (Martin *et al.* 2005).

Phylogenies were estimated separately for each sequenced region. Indels were not recoded. Alignments were previously collapsed to contain only unique sequence types/alleles in COLLAPSE 1.2. The best-fit model of DNA substitution for each sequenced region was selected by hierarchical Akaike information criterion (AIC) (Posada and Buckley 2004) in MODELTEST 3.7 (Posada and Crandall 1998) based on a neighbour-joining tree and Jukes Cantor distances. The selected models of sequence evolution (*nad5*: TrN, *ATPSα*: K81uf, *SRP54*: K81uf+G) were used in maximum likelihood (ML) phylogeny inference in PAUP* 4.0B10 (Swofford 2002), employing heuristic searches with random stepwise addition replicates (100 and 1000 for nuclear and mitochondrial markers, respectively) and tree bisection-reconnection (TBR) branch swapping. Results were summarised as 50% majority rule consensus trees, rooted using sequences obtained from other sampled coral genus (data unavailable for the *ATPSα*). Robustness of the nodes was assessed by non-parametric ML-bootstrap analysis (500 and 1000 pseudoreplicates, respectively), performed using the same models of evolution, heuristic replicates (10 and 100, respectively) with random stepwise addition and nearest-neighbour interchange (NNI) branch swapping.

Population genetics

Analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was used to test the significance of population structure among and within *Madracis* taxa, assuming each putative species as a population. The AMOVA estimates an analogue of Wright's F_{ST} (Wright 1965), which takes into account the number of mutations between molecular haplotypes. Pairwise F_{ST} and their significance were calculated to estimate morphospecies differentiation. All calculations were performed using genetic distances corrected by a Kimura two-parameter model of evolution in ARLEQUIN 3.1, a Gamma distribution shape parameter as previously determined by MODELTEST 3.7 and with 1000 random permutations for significance tests. Inbreeding coefficient of individuals within populations (F_{IS}) provided insight on possible departures of genotype frequencies from Hardy-Weinberg Equilibrium for each species (HWE; positive values represent heterozygote deficits). HWE was further tested using a Markov chain approach that estimates the probability of the current allelic configuration of the genotypes.

RESULTS

Sequence alignments and features

Table 6.1 gives an overview of the symbiont types and available sequences of all samples

used in our study. Successful PCR amplification and sequencing accounted for 94-95% of the zooxanthellate *Madracis* samples (115 out of 121 for mtDNA, 73 out of 78 for both *ATPSα* and *SRP54*). Final sequence alignments were 565, 342 and 202 bp-long, for respectively, mtDNA, *ATPSα* and *SRP54*. Unusual longer-sized fragments were detected for some samples and their presence confirmed by PCR reamplification, DGGE separation and sequencing. The fact that preliminary PCR optimization under lower temperatures did not promote the amplification of these longer fragments suggests that they did not originate from multiple loci, but represent cases of length polymorphism in the same loci. This accounted for two samples in mtDNA (*c*. 800-bp distinct haplotypes in a *M. formosa* and a *S. siderea*) and one sample in *SRP54* (*S. siderea* displaying heterozygous genotype, with a 400-bp long allele; the short one being used as outgroup). In *ATPSα*, long fragments (*c*. 700-bp) were less uncommon, being present in one homozygous *M. senaria* and one heterozygous *M. formosa* (long alleles were distinct). *ATPSα* length polymorphism is recurrent in at least two more *M. senaria* samples, although not confirmed due to sequencing failure. All these longer alleles were excluded from analysed sequence alignments and respective samples excluded from all statistics using diploid genotypic data.

A concise description of the sequenced regions is provided in Table 6.2. There was little difference in base composition between the sequenced regions, although mtDNA had a slight bias toward A and T and away from G. On the other hand, the nuclear *ATPSα* has a deficit of A and shows the lowest AT content. No differences in base composition are displayed between the six recognised *Madracis* species (data not shown). However, the two nuclear markers differ by more than one order of magnitude from mtDNA in terms of variable positions, nucleotide diversity and sequence distances. With respect to the two nuclear regions, *SRP54* is more variable, more diverse and shows larger distances than *ATPSα*. The number of indels in the alignment is larger for *SRP54* than *ATPSα*, and absent for mtDNA.

Recombination was not detected within nuclear loci. No significant deviation from neutral expectations was found in either region for Tajima's *D*, a neutrality test based on nucleotide diversity and number of segregating sites. However, Fu's *Fs*, a test based on the number of haplotypes in a population given observed nucleotide diversity, indicated a single significant deviation from neutrality for mtDNA. This suggests that some non-random processes may be playing a role in the evolution of the region. This interpretation is strengthened by the fact that the two parsimonious substitutions detected are displayed in the exon region, one of them constituting a non-synonymous change.

After collapsing all the sequences obtained for each region, 9 unique haplotypes remained for *nad5*, and respectively 34 and 61 unique alleles for *ATPSα* and *SRP54*. These numbers would be narrowed down to 6, 31 and 58 if sequence missing data was not considered.

Phylogenetic inference

Figure 6.2 shows the ML phylogenetic topologies inferred for each of the three studied DNA regions. The *nad5* tree was rooted with a *Pocillopora eydouxi* sequence retrieved from GenBank (DQ351263). The mtDNA topology (Figure 6.2a) is characterised by the existence of one robust clade that separates the putative species into two groups. This separation matches the ecological distribution of the genus, with species that are typical of shallow waters clustering together (*M. mirabilis* and *M. decactis*), while deep water or depth generalist species form the

Table 6.1 Description of the samples used in this study, including species, location and depth, symbiont type, colony colour morph (for *M. pharensis* only), sample code and available sequences.
[a] Symbiont rDNA ITS2 types; NA = symbiont type not determined; note that mixed symbiont populations were detected for some samples (e.g. B7&B15).
[b] Colour morph for *M. pharensis* colonies; NA = colour not registered.

Species	Location	Depth (m)	Symbiont[a]	Colour[b]	Sample Code	Sequences
M. carmabi	Buoy One, Curaçao	28	B7		74	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	27	B7		115	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	41	B7		424	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	39	B7&B15		479	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	40	B7		480	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	41	B7		600	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	39	B7		601	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	40	B7		602	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	40	B7		603	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	41	B7&B15		604	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	12	B7	Brown	607	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	12	B7	Purple	608	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	12	B7	Brown	609	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	12	B7	Brown	610	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	12	B7	Brown	611	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	23	B15	Green	612	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	24	B15	Brown	613	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	23	B7	Brown	614	*nad5, ATPSα*
M. pharensis	Buoy One, Curaçao	23	B7	Brown	615	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	24	B7	Purple	616	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	40	B15	Brown	627	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	40	B15	Green	628	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	40	B15	Green	629	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	10	B7		633	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	11	B13		634	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	10	B13		635	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	8	B13&B7		636	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	9	B13		637	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	10	B13		638	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	10	B13		639	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	12	B7		640	*nad5, ATPSα*
M. mirabilis	Buoy One, Curaçao	11	B13		641	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	20	B13		642	*nad5, ATPSα, SRP54*
M. mirabilis	Buoy One, Curaçao	19	B13&B7		643	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	12	B7		654	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	10	B7		655	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	12	B7		656	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	13	B7		657	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	13	B7		658	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	25	B7		659	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	23	B7		660	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	10	B7		670	*nad5, SRP54*
M. pharensis	Playa Kalki, Curaçao	13	B7	Brown	672	*nad5*
M. pharensis	Playa Kalki, Curaçao	13	B7	Brown	673	*nad5*
M. pharensis	Playa Kalki, Curaçao	13	B7	Brown	674	*nad5*
M. pharensis	Playa Kalki, Curaçao	13	B7	Brown	675	*nad5*
M. pharensis	Playa Kalki, Curaçao	13	B7	Green	676	*nad5*
M. pharensis	Playa Kalki, Curaçao	13	B7	Brown	677	*nad5*
M. senaria	Playa Kalki, Curaçao	13	NA		678	*nad5*
M. senaria	Playa Kalki, Curaçao	13	B7		679	*nad5*
M. senaria	Playa Kalki, Curaçao	13	NA		680	*nad5*
M. senaria	Playa Kalki, Curaçao	13	B7		681	*nad5*
M. senaria	Playa Kalki, Curaçao	13	B7		682	*nad5*
M. senaria	Playa Kalki, Curaçao	13	B7		683	*nad5*
M. mirabilis	Buoy One, Curaçao	10	B13		721	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	42	B15		876	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	40	B15		877	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	39	B7		879	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	26	B7		880	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	23	B7		881	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	26	B7		882	*nad5, ATPSα, SRP54*

Table 6.1 (*continued*)

Species	Location	Depth (m)	Symbiont[a]	Colour[b]	Sample Code	Sequences
M. carmabi	Buoy One, Curaçao	23	B7		888	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	21	B7		889	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	11	B7		890	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	11	B7		891	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		892	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	30	B7		893	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	26	B7		894	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	32	B7		895	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	9	B7		896	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	9	B7		897	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		898	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	11	B7		899	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		900	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	10	B7		901	*nad5, ATPSα, SRP54*
M. senaria	Buoy One, Curaçao	10	B7		902	*nad5, SRP54*
M. carmabi	Buoy One, Curaçao	20	B7		903	*nad5, ATPSα*
M. decactis	Buoy One, Curaçao	11	B7		904	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		905	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	28	B15	Green	910	*nad5, ATPSα, SRP54*
M. carmabi	Buoy One, Curaçao	27	B7		922	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	12	B7		923	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		924	*nad5, ATPSα, SRP54*
M. decactis	Buoy One, Curaçao	10	B7		925	*nad5, ATPSα, SRP54*
M. formosa	Buoy One, Curaçao	56	B15		951	*nad5, ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	56	B15	NA	952	*nad5*
M. pharensis	Buoy One, Curaçao	58	B15	NA	953	*ATPSα, SRP54*
M. pharensis	Buoy One, Curaçao	57	B15	NA	954	*nad5, ATPSα*
M. pharensis	Buoy One, Curaçao	56	B15	NA	955	*nad5, SRP54*
M. formosa	Buoy One, Curaçao	57	B15		956	*SRP54*
M. mirabilis	Aruba	10	B7		958	*nad5*
M. mirabilis	Aruba	12	B13		959	*nad5*
M. mirabilis	Aruba	8	B13		960	*nad5*
M. mirabilis	Aruba	7	B13		961	*nad5*
M. decactis	Aruba	16	NA		962	*nad5*
M. decactis	Aruba	15	NA		963	*nad5*
M. decactis	Aruba	13	NA		964	*nad5*
M. pharensis	Aruba	18	B7	NA	965	*nad5*
M. decactis	Aruba	18	NA		966	*nad5*
M. decactis	Aruba	16	NA		967	*nad5*
M. pharensis	Aruba	19	B15	NA	968	*nad5*
M. carmabi	Aruba	19	B7		969	*nad5*
M. pharensis	Aruba	11	B7	NA	970	*nad5*
M. pharensis	Aruba	12	B7	NA	971	*nad5*
M. senaria	Aruba	17	NA		972	*nad5*
M. senaria	Aruba	18	NA		973	*nad5*
M. senaria	Aruba	18	NA		974	*nad5*
M. formosa	Bermuda	55	NA		B1	*nad5*
M. decactis	Bermuda	50	NA		B2	
M. mirabilis	Bermuda	6	NA		B3	*nad5*
M. mirabilis	Bermuda	6	NA		B4	*nad5*
M. decactis	Bermuda	6	NA		B5	*nad5*
M. senaria	Bermuda	6	NA		B6	
M. mirabilis	Trinidad & Tobago	10	NA		T1	
M. mirabilis	Trinidad & Tobago	10	NA		T2	
M. mirabilis	Trinidad & Tobago	10	NA		T3	*nad5*
M. mirabilis	Trinidad & Tobago	9	NA		T4	*nad5*
M. mirabilis	Trinidad & Tobago	9	NA		T5	*nad5*
M. mirabilis	Trinidad & Tobago	8	NA		T6	*nad5*
M. mirabilis	Trinidad & Tobago	8	NA		T7	*nad5*
M. mirabilis	Trinidad & Tobago	8	NA		T8	*nad5*
M. myriaster	Buoy One, Curaçao	300			JK1	*SRP54*
S. siderea	Buoy One, Curaçao	19	NA		RH30	*nad5, SRP54*

Table 6.2 Sequence statistics for each of the three loci. Total number of sequences in final alignments, total alignment length (number of sites), number of variable, parsimony informative and indel sites, number of unique haplotypes or alleles, AT content, nucleotide diversity, maximum uncorrected p-distance and neutrality test results for each sequenced fragment. Note from Table 6.1 that in the case of *nad5* the samples were not only from Buoy One (Curaçao), but also from other locations.

	nad5	*ATPSα*	*SRP54*
Number of sequences	115	142	146
Number of sites	565	342	202
Number of variable sites	4	48	68
Number of parsimony sites	2	33	57
Number of sites with indels	0	12	84
Number of unique haplotypes or alleles	9	34	61
% AT (SE)	71.77 (0.02)	66.18 (0.03)	69.64 (0.16)
Nucleotide diversity (SE)	0.001 (0.001)	0.021 (0.004)	0.072 (0.010)
Maximum uncorrected p-distances (%)	0.39	5.42	17.12
Tajima's *D* (P-value)	- 0.258 (0.458)	- 0.576 (0.331)	- 0.136 (0.509)
Fu's *Fs* (P-value)	inf. neg. (0.000)	- 4.128 (0.196)	- 7.079 (0.138)

other group (*M. senaria*, *M. carmabi* and *M. formosa*). Although the mtDNA sequences are mostly invariant among conspecifics, *M. pharensis* represents an exception and *M. pharensis* haplotypes are present in both groups. A closer look reveals that there is a clear distinction between *M. pharensis* samples originating from the shallower ($\leq$ *c.* 23 m) and from the deeper reef habitats ($\geq$ *c.* 23 m), and that they group with the other *Madracis* species according to the same ecological pattern. Interestingly, the *M. pharensis* mtDNA branching matches exactly with symbiont type harboured (*see* also Table 6.1): ITS2 type B7 in the shallows and B15 in the deep habitats, representing a symbiont ecological zonation previously described (Frade *et al.* 2008c). There is yet another representative haplotype for *M. pharensis*, mostly present in the other Curaçao location, Playa Kalki.

Samples originating from geographical locations other than Curaçao (Bermuda, Trinidad and Tobago, Aruba), remarkably showed no intraspecific mtDNA variation (with a few exceptions, *see* Figure 6.2a), fitting the two general groups already described and strengthening the idea that the genetic structure of *M. pharensis* is an exception within the genus. Any doubt about the correct identification of *M. pharensis* arising from the mtDNA tree is quickly removed when examining the structure of the *ATPSα* topology (Figure 6.2b), for which *M. pharensis* shows unique, non-shared, alleles. Phylogenetic inference based on *ATPSα* consistently indicates polyphyletic taxa. There are, however, two exceptions. *Madracis senaria* forms a well supported clade. It is the only monophyletic species within the genus. *Madracis carmabi* is nearly monomorphic, with the exception of distinct alleles present in two samples. The monomorphic branch suggests specific low genetic diversity. The other well supported clades form mixed assemblages of *M. mirabilis* and *M. formosa*, *M. decactis* and *M. pharensis*, or *M. pharensis* and *M. mirabilis*. Similarly to the *ATPSα* analysis, *SRP54* (Figure 6.2c) provides low resolution for inferring molecular phylogenies, with several small and mixed clades, usually combining *M. pharensis*, *M. decactis*, *M. formosa* and *M. carmabi*. The polyphyletic nature of *Madracis* taxa is not even challenged by *M. senaria*, which clusters in two separate but restricted groups, one monophyletic and the other paraphyletic (with a *M. decactis* allele).

The azooxanthellate species *M. myriaster* appears to be phylogenetically very close to the other *Madracis* species. The heterozygote colony sampled shares an allele with a deep *M. pharensis*

a)

Figure 6.2 Maximum likelihood trees of the *Madracis* genus for (**a**) the mitochondrial *nad5* intron, (**b**) the nuclear *ATPSα* intron and (**c**) the nuclear *SRP54* intron. Values above the branches are bootstrap values for 1000, 500 and 500 replicates, respectively. For full colour version *see* Appendix (page 134).
Sequence codes refer to: Species[a]DepthLocation[b]_Sample[c].
[a]Species codes: Mmir, *M. mirabilis*; Mfor, *M. formosa*; Mdec, *M. decactis*; Msen, *M. senaria*; Mpha, *M. pharensis*; Mmyr, *M. myriaster*; Ssid, *Siderastrea siderea*.
[b]Location codes: Bu1, Buoy One (Curaçao); Kal, Playa Kalki (Curaçao); Ber, Bermuda; Aru, Aruba; Tri, Trinidad & Tobago. Location not indicated for *ATPSα* and *SRP54* topologies (all samples from Buoy One, Curaçao).
[c] Sample codes as in Table 6.1 ("a" and "b" added to distinguish alelles in heterozygotes).

colony, while the other allele is included in a well supported clade comprising alleles from all the other taxa except *M. mirabilis*.

Population genetics

AMOVAs were carried out for the whole sample set assuming each putative species as a population (Table 6.3). For *ATPSα*, the genetic variation was hierarchically structured, with about 52% distributed among putative species, about 23% among individuals within the species and 26% within individuals. For *SRP54* the distribution of the genetic variation followed the opposite trend, with only about 13% attributable to both variation among species and among individuals within the species, and most of the variation (about 73%) occurring within individuals. Fixation indices showed significantly high genetic divergence at all hierarchical levels for both studied DNA regions, with the highest structuring within individuals and the lowest among species and among individuals within the species. Thus, AMOVA for both nuclear introns indicates statistical divergence (at the 0.1% significance level) among putative *Madracis* species. Pairwise comparisons of genetic differentiation between the six *Madracis* species based on F_{ST} values (Table 6.4) confirm similar overall divergence but highlights important exceptions. For *ATPSα*,

b)

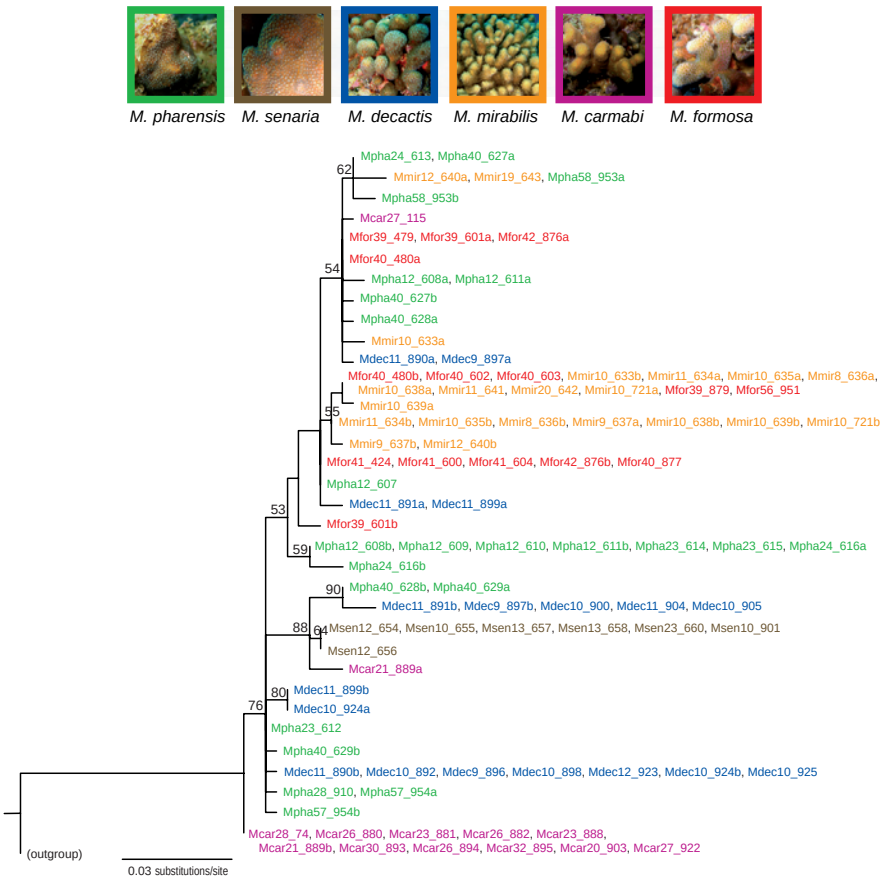

Figure 6.2 (*continued*) For full colour version *see* Appendix (page 135).

all pairwise comparisons were highly statistically significant (at the 0.1% significance level), except that of the pair *M. mirabilis* / *M. formosa* (only divergent at the 5% significance). In fact, for this region, these are the only two species that profusely share alleles, in this case a single allele which is present in more than 40% of the locus' copies for each species. Besides this, there is only another single case of allele sharing, involving two *M. mirabilis* and one *M. pharensis* colonies, which however does not cause a drop of the statistically significant divergence between the two species. The other nuclear marker, *SRP54*, shows recurring allele sharing (9 alleles shared out of a total of 61) and more evenly distributed allele frequencies among the allele pool than *ATPSα* (data not shown). All *Madracis* taxa except *M. senaria* are involved in *SRP54* allele sharing, with shared alleles accounting for a total of the locus's copies of about 30% for *M. mirabilis*, 73% for *M. formosa*, 19% for *M. decactis*, 27% for *M. carmabi* and 27% for *M. pharensis*. Almost all F_{ST} pairwise comparisons for *SRP54* are significant, once again confirming the genetic differentiation between most *Madracis* species. However, most pairwise comparisons are only significant at the 5% level, suggesting gene exchange may be occurring between some of these taxa, something highly expected in the case of *M. carmabi* / *M. formosa* (non-significant comparison).

Genetic differentiation among *Madracis* taxa suggested by both DNA regions studied (and prominently expressed in *ATPSα*) is evidence for long term limits to gene flow, but could at the

c)

Figure 6.2 (*continued*) For full colour version *see* Appendix (page 136).

same time be caused by natural selection (or even by recent changes in population sizes). For this reason, neutrality was again tested, this time separately for each putative species, using the tests Tajima's *D* and Fu's *Fs* (Table 6.5). The only significant deviation from neutral expectations was found by Tajima's *D* for *ATPSα* sequences in *M. carmabi*, suggesting that non-random processes may play a role in the evolutionary dynamics of this taxon.

Observed heterozygosity levels are shown in Table 6.6, as determined by ARLEQUIN. Only rarely did the heterozygosity levels fulfil the expectation for HWE predicted under conditions of random mating. Inbreeding coefficients (F_{IS}) confirmed these results. Overall, *ATPSα* shows a higher heterozygote deficit than *SRP54*, with about 37% against about 81% of heterozygote samples (colonies), respectively. These estimations only include samples for which the zygosity pattern inferred from the DGGE band migration pattern was further confirmed by sequencing, and exclude two samples for which *ATPSα* long introns were detected (including them resulted in identical heterozygosity level). For *ATPSα*, only *M. mirabilis* did not show a departure of genotype frequencies from HWE. All other five species showed significant heterozygote deficits,

Table 6.3 Results of AMOVA analysis for *ATPSα* and *SRP54* nDNA sequences. Statistical significance (* $P < 0.05$; ** $P < 0.001$) is based on 1000 random permutations.

		ATPSα				*SRP54*		
Source of variation	d.f.	Variance components	% of variation	Fixation indices	d.f.	Variance components	% of variation	Fixation indices
Among species	5	2.094	51.55	Φ_{ST}: 0.516 **	5	1.130	13.40	Φ_{ST}: 0.134 **
Among individuals within species	65	0.932	22.94	Φ_{IS}: 0.473 **	67	1.116	13.24	Φ_{IS}: 0.153 *
Within individuals	71	1.036	25.52	Φ_{IT}: 0.745 **	73	6.186	73.36	Φ_{IT}: 0.266 **

Table 6.4 Pairwise F_{ST} values among *Madracis* species calculated from *ATPSα* (below diagonal) and *SRP54* (above diagonal) nuclear markers. Statistical significance (* $P < 0.05$; ** $P < 0.001$) is based on 1000 random permutations.

	M. mirabilis	*M. formosa*	*M. decactis*	*M. senaria*	*M. carmabi*	*M. pharensis*
M. mirabilis	-	0.231 **	0.089 *	0.301 **	0.176 **	0.067 **
M. formosa	0.064 *	-	0.133 *	0.195 *	0.014	0.157 *
M. decactis	0.510 **	0.477 **	-	0.167 **	0.068 *	0.045 *
M. senaria	0.865 **	0.892 **	0.444 **	-	0.183 *	0.241 **
M. carmabi	0.728 **	0.727 **	0.346 **	0.807 **	-	0.100 *
M. pharensis	0.395 **	0.337 **	0.190 **	0.589 **	0.410 **	-

especially *M. formosa* and *M. pharensis* (0.1% significance level). For *SRP54*, *M. decactis* and *M. senaria* showed evidence for heterozygote excess, whereas genotype frequencies revealed significant deficits for *M. mirabilis*, *M. carmab*i and *M. pharensis*. High F_{IS} values and significant heterozygote deficits are persistent for both studied regions in *M. pharensis* and *M. carmabi*, suggesting high levels of inbreeding among conspecifics and possibly self-fertilization.

To address potential differentiation between the two depth groups of *M. pharensis*, the population genetics approach was repeated for the whole nuclear DNA datasets, this time assuming two *M. pharensis* populations, which correspond to the two main branches yielded by the mtDNA phylogeny. Neutrality was not rejected and the overall AMOVA results were identical to the previous approach, highlighting the statistical divergence among *Madracis* taxa (*ATPSα*: Φ_{ST} = 0.538, d.f. = 6, $P < 0.001$, $n = 71$; *SRP54*: $\Phi_{ST} = 0.144$, d.f. = 6, $P < 0.001$, $n = 73$). However, the pairwise comparisons offered new information. The two *M. pharensis* groups were statistically divergent from each other (*ATPSα*: $F_{ST} = 0.280$, $P < 0.001$; *SRP54*: $F_{ST} = 0.136$, $P = 0.003$) and shared alleles are restricted to one single SRP54 case. For *ATPSα*, both *M. pharensis* depth groups were highly divergent from all other *Madracis* taxa ($P < 0.001$), except for a weaker but still significant divergence between the deep population and *M. decactis* ($F_{ST} = 0.146$, $P = 0.009$). For *SRP54*, most statistical comparisons were also kept identical to the previous approach, except for the absence of divergence between *M. carmabi* and the deep *M. pharensis* ($F_{ST} = 0.053$, $P = 0.092$). Comparison to the shallow group was still significant ($F_{ST} = 0.180$, $P = 0.003$).

Another AMOVA was run to address the genetic differentiation between *M. pharensis* colour morphs (green vs. brown). Divergence was only slightly statistically significant for *ATPSα* ($\Phi_{ST} = 0.255$, d.f. = 1, $P = 0.031$, $n = 12$) and non-existent for *SRP54* ($\Phi_{ST} = 0.018$, d.f. = 1, $P = 0.768$, $n = 11$).

DISCUSSION

The multilocus genotyping approach of this study has proven helpful in exploring species barriers in the brooding coral genus *Madracis*. It has demonstrated that, while morphospecies have close genetic affiliations, they do represent significantly distinct gene pools. Introgressive hybridization as well as natural selection have most likely played important roles in shaping the genome of these species.

Madracis genetic relationships

The phylogenetic topologies inferred by the three DNA regions studied are not congruent with each other. Phylogenetic analyses did not recognize reciprocal monophylies, though alleles were in general not shared between the *Madracis* putative species, and the *ATPSα*-based population genetics approach suggests that most morphospecies constitute genetically distinct lineages. However, *SRP54* provides evidence for gene exchange among *Madracis* taxa. To understand the species relationships in the *Madracis* genus one needs to address the different evolutionary pressures acting on the DNA regions studied, their great discrepancies in sequence variation, and their level of phylogenetic inference.

The first pattern revealed by mtDNA *nad5* region is that the *Madracis* taxa are highly similar. Although this marker is considered of little use for within-species comparisons (Concepcion *et al.* 2006), the variation found for the *nad5* non-coding region is virtually non-existent and thus the question has to be raised whether *Madracis* morphospecies are "real" species, according to the phylogenetic species concept (Donoghue 1985). Another interesting issue brought up by

Table 6.5 Results of the neutrality tests Tajima's *D* and Fu's *Fs* for each species and each nuclear region. Statistical significances (* P < 0.05; ** P < 0.001) are based on 1000 simulations.

	ATPSα		*SRP54*	
	Tajima's *D*	Fu's *Fs*	Tajima's *D*	Fu's *Fs*
M. mirabilis	-0.537	2.208	-0.199	0.404
M. formosa	-0.233	2.723	3.177	21.736
M. decactis	0.893	6.729	-0.499	0.132
M. senaria	0.000	1.251	1.382	8.270
M. carmabi	-1.762 *	5.276	0.956	4.454
M. pharensis	-0.204	-1.452	-0.926	-1.113

Table 6.6 *Madracis* inbreeding coefficient of individuals (F_{IS}) within species and Hardy-Weinberg equilibrium test (HWE: observed heterozygosity / expected heterozygosity; sample size indicate in parentheses) for the *ATPSα* and *SRP54*. Statistical significances (* *P* < 0.05; ** *P* < 0.001) are based, for F_{IS}, on 1000 random permutations.

	ATPSα		*SRP54*	
	F_{IS}	HWE	F_{IS}	HWE
M. mirabilis	0.333	0.75 / 0.75 (12)	0.161	0.82 / 0.91 (11) *
M. formosa	0.525 **	0.27 / 0.73 (11) **	0.028	0.69 / 0.82 (13)
M. decactis	0.476 *	0.38 / 0.70 (13) *	-0.077	1.00 / 0.96 (13)
M. senaria	(-9.999) **	(0.00 / 0.26) (7)	0.182	0.90 / 0.78 (10)
M. carmabi	0.683 *	0.08 / 0.24 (12) *	0.372*	0.73 / 0.92 (11) *
M. pharensis	0.430 **	0.50 / 0.87 (16) **	0.243*	0.73 / 0.97 (15) *

the mtDNA data is that even the geographically distant populations (Bermuda, Trinidad and Tobago) are invariant in their sequences. Anthozoan mtDNA is believed to be associated with a mismatch repair mechanism that may explain the low levels of variability in mtDNA compared to nuclear markers (van Oppen *et al.* 1999; Shearer *et al.* 2002; Huang *et al.* 2008a). This has greatly limited the ability to do phylogeography and phylogenetics in this group of organisms. Another, non-mutually exclusive, explanation for the low levels of genetic diversity of mtDNA haplotypes is that there may be factors other than recent common ancestry acting in this region. Possible candidates are the selective pressure of natural selection or severe bottleneck events during the evolutionary history (Avise *et al.* 1987). These would be even more drastic in the mitochondrial genome due to the smaller effective population size of the matrilineal transmitted haploid genome. In fact, Fu's *Fs* (Fu 1997) indicated a significant deviation from neutrality for mtDNA, evidence strengthened by the coding location of the only two parsimonious substitutions detected. This transcriptional difference stresses that this mitochondrial region appears to be under selection. The clear pattern of mtDNA phylogenetic separation matches the ecological (depth) distribution of the genus, suggesting that the species are ecologically differentiated and may be under influence of disruptive selection. The mtDNA gene in cause, *nad5* (or NADH ubiquinone oxidoreductase subunit 5, Wissinger *et al.* 1988), is involved in a multisubunit enzyme complex of the mitochondrial inner membrane that catalyzes the first step in mitochondrial respiration, which is a temperature dependent process. This is just one possible functional link to an eco-evolutionary pressure with differential effects along the depth gradients.

Both nuclear DNA markers show major amounts of paraphyly within the *Madracis* genus. However, there was no congruence between the two in resolving phylogenetic species. The A*TPSα* ML bootstrap analysis recovered the monophyly of *M. senaria*. For *SRP54*, although polyphyletic, this species showed the lowest sequence variation, being restricted to two well supported clades, unlike all the other taxa. Besides, *M. senaria* was the only taxon for which allele sharing was absent in both nuclear regions studied. *Madracis senaria* was found by Diekmann *et al.* (2001) to be the only *Madracis* taxon forming a well supported monophyletic clade in a phylogenetic analysis using rDNA ITS. These evidences taken together constitute sufficient argument for considering *M. senaria* the only genetically isolated species in the genus *Madracis*.

Although the phylogenetic analyses did not resolve further species-level relationships, the population genetics approach did find statistically distinct lineages. AMOVAs indicate, for both nuclear introns, overall statistical divergence among putative *Madracis* species. Perhaps the most remarkable result is the genetic divergence between the two *M. pharensis* depth groups. Since this divergence is similar to that measured for well established interspecific comparisons (e.g. *M. senaria* and *M. formosa*), one has to consider the possibility that these two groups represent distinct populations or even subspecies. This divergence suggests the hypothetical presence of further unknown genetic pools in even deeper reefs (Hinderstein *et al.* in press). Phenotypically, these two *M. pharensis* populations differ in the symbiont signature, with the shallow colonies harbouring exclusively rDNA ITS2 type B7 and the deep colonies hosting B15 type, a pattern that is known to correspond to physiological competence and adaptation to depth (Frade *et al.* 2008a). Colour morphs of *M. pharensis* are distributed according to depth (Vermeij and Bak 2003) and in the present study the sample sizes did not allow isolating the colour morph effect (from depth) on genetic variation. Nevertheless, green and brown colonies

did not show consistent genetic differentiation. This result suggests that colour in scleractinians is not underpinned by phylogenetic difference (Mackenzie *et al.* 2004).

Some *Madracis* taxa showed low nDNA divergence in the population genetics approach, suggesting gene exchange. For *ATPSα*, the pairs *M. decactis* / deep *M. pharensis* and *M. mirabilis* / *M. formosa* are not highly statistically divergent. For *M. mirabilis* and *M. formosa* this is a somewhat surprising result considering the non-overlapping depth distributions (Vermeij and Bak 2003) and the differences in morphology (e.g. septal structures, Wells 1973a) and symbiont signatures (Frade *et al.* 2008c) of these species. Besides, the present study does not confirm *M. mirabilis* as a monophyletic group, a result contrasting with that of Diekmann *et al.* (2001). *SRP54* data suggests extensive gene exchange may occur especially between species such as *M. formosa*, *M. carmabi* and (the deep) *M. pharensis*. This is similar with the rDNA ITS-based paraphyletic species complex described by Diekmann *et al.* (2001) for the same species plus *M. decactis* (at the time *M. carmabi* had still not been described as a separate species, being identified as *M. decactis*). *Madracis myriaster*, the only exclusively azooxanthellate species in the *Madracis* genus appears to be phylogenetically close to this species complex.

Introgressive hybridization

The non-monophyly of nearly all *Madracis* taxa, in combination with an incongruence between nuclear and mitochondrial DNA markers, and the high levels of shared polymorphism, suggest that either introgressive hybridization has occurred or that lineage sorting of ancestral polymorphism is incomplete. An old (15-11 million years) fossil record of at least some of the species involved, *M. mirabilis* and *M. decactis* (Budd *et al.* 1994; Budd *et al.* 1995; Budd and Johnson 1999), suggests that hybridization is likely to have played a role, for the evolutionary time that passed since speciation took place would have been enough to solve lineage sorting (Diekmann *et al.* 2001). As such, the non-monophylies are likely due to hybridization.

Another argument for hybridization within the genus is the case of *M. pharensis*, for which a different mtDNA haplotype was found to be predominantly present in another sampling location, Playa Kalki, located 30 Km west and downstream from the main collection site, the Buoy One reef. This haplotype differs by a single silent substitution in the exon region of the *nad5* (and as such we assume it is selectively neutral). The fact that a species with a relatively old (1.5 million years) fossil record as *M. pharensis* shares genetic material more frequently with other species with which it co-exists in sympatry (e.g. *M. carmabi*), than with its own conspecifics located only a few Km apart, suggests that there is hybridization going on and that spatial scales may play a role therein.

Remarkably, a similar situation was found on a smaller, depth-based spatial scale at the Buoy One location. Deep water *M. pharensis* are genetically closer to non-conspecific *Madracis* originating from the same depth (*M. carmabi*), than to conspecifics inhabiting the shallower depths of the same reef. Such a well defined spatial divergence could be associated with vertical gradients of temperature and light. In fact, the depth marking the genetic divergence of *M. pharensis* matches with bathymetric data of the thermocline position in Curaçao (Bak *et al.* 2005; Frade *et al.* 2008c). Thermocline position may indicate not just the presence of lower temperatures but relate to other relevant water mass characteristics, such as nutrient concentrations (Leichter *et al.* 1996). Thermoclines may function as a recurrent environmental barrier that while preventing continuous cross-fertilization between *M. pharensis* populations at different depths, stresses the

homogenising effect of introgression bridges between species. An interesting but speculative hypothesis is that the deep and shallow populations of *M. pharensis* get isolated from each other not directly by the effect of abiotic barriers coupled to depth, but rather by the acclimatisation capabilities of their symbionts (Iglesias-Prieto *et al.* 2004). Physiological competence of algal symbionts (Frade *et al.* 2008a) is known to relate to coral processes such as juvenile growth and survival (Little et al. 2004; Abrego et al. 2008; Gomez-Cabrera et al. 2008). This is a pathway that can possibly lead to the disruptive selection and divergence of host taxa facilitating coevolution between corals and their symbionts.

Some *Madracis* reproductive biology aspects add-up as arguments for possible introgressive hybridization. *Madracis* species are simultaneous hermaphrodites and show no temporal reproductive isolation, with mature gametes being present mostly in the autumn, preceding maximum planulae release in the same season (Vermeij *et al.* 2003b,2004). *Madracis senaria*, however, has a lunar pattern of planulae release superimposed on the seasonal cycle and it spawns more planulae than all other species. Note that in the case of *Madracis* the time from fertilization to planulae release is known to be short (Vermeij *et al.* 2004) and so, most likely, the fertilization is also periodic in *M. senaria* and distinct from the other species. This reproductive difference could be enough to generate the genetic differentiation here confirmed. In fact, this is the only genetic monophyletic *Madracis* taxon according to the present study and this monophyly was also indicated by rDNA ITS phylogeny (Diekmann *et al.* 2001). The other monophyletic lineage suggested by Diekmann *et al.* (2001), *M. mirabilis*, also differs in fecundity, releasing the least planulae in the genus, and it spreads mostly by colony fragmentation (Vermeij *et al.* 2003b), unlike the other species in the genus. There are also relevant differences in oocyte sizes: *M. mirabilis* and *M. senaria* show large yolk reserves (Vermeij *et al.* 2003b), an indication that the planulae may travel larger distances. This may relate to less chance for isolation of populations when facing stronger geographical barriers. Consequently this may explain the lower genetic structure shown in the present study for these two species. Other reproductive attributes may also play a role in the hybridization success. For instance, in the Caribbean genus *Montastraea* (in Panama, Fukami *et al.* 2004a; Levitan *et al.* 2004), species boundaries are maintained by a suite of isolating barriers. The two species that have the more compatible gametes and are most closely related genetically (*Montastraea franksi* and *Montastraea annularis*) show strong temporal isolation, whereas the species that spawn simultaneously (*M. annularis* and *Montastraea faveolata*) have incompatible gametes and are genetically further apart.

Overall, the fact that taxa forming distinct genetic clusters also differ in reproductive traits and symbiont signatures, and that those others which are similar in their biology form more homogeneous groups, is consistent with the suggested introgression within the genus.

A hybrid origin for *Madracis carmabi*?

M. carmabi, a recently described species (Vermeij *et al.* 2003a) for which little is known about its reproductive traits, has been suggested to represent a hybrid combining characteristics of *M. decactis* (10 primary septa) and *M. formosa* (branching morphology). The *ATPSα* data of *M. carmabi* show very low genetic diversity. Accordingly, *M. carmabi ATPSα* sequences constitute the only significant deviation from neutral expectations within the genus, as tested by Tajima's *D*, suggesting that non-random processes may play a role in the evolution of the taxon. Such a process could be a recent change in population size, exactly what is to be expected if *M. carmabi* would be the product of recent hybridization. This hypothesis matches with absence of

M. carmabi in the fossil record. Further genetic studies will be needed to confirm the hypothetic hybrid species status of *M. carmabi*. However, the *SRP54* data suggests that the species maintains gene exchange with *M. formosa* and with the deep population of *M. pharensis* and that this could be its parental line, instead of *M. formosa* and *M. decactis* (Vermeij *et al.* 2003a). Overall, this study provides evidence that could support a hybrid origin of *M. carmabi*. Interestingly, there is also broad evidence for inbreeding between conspecifics among *Madracis* taxa. Both markers showed heterozygote deficits but these results are especially persistent for *M. pharensis* (the deep water population) and *M. carmabi*, suggesting that these species may be experiencing high levels of self-fertilization.

A role for permeable species barriers

The evidence for gene exchange involving *M. formosa* indicated by *SRP54* is repeated for *ATPSα*. In fact, this species does not seem to constitute a highly significantly divergent lineage. Even if one cannot exclude a recent origin with incomplete lineage sorting for this species (fossil record is also unknown), it is interesting to consider that *M. formosa* is more permeable to gene exchange than the other species. A reasonable cause would be the low density with which this species occurs, which could preclude successful mating between conspecifics, leaving an open door for cross-fertilization events. Hypothetically lower reproductive barriers in *M. formosa* would more likely be related to receiving sperm from non-conspecific colonies than to fertilization of eggs located in non-conspecific colonies. However, not much is known on sperm cloud behaviour for brooding corals. It is important to note that due to the brooder reproductive nature of *Madracis*, it is virtually impossible to perform *in vitro* breeding trials that could set tests to our hypothesis on species barriers between *Madracis* species.

There is only one other molecular study suggesting hybridization among brooding corals. In an allozyme study in Indo-Pacific scleractinian species, Miller and Ayre (2004) found that at the edge of their geographical ranges, on high latitude reefs of Lord Howe Island, the brooding species *Pocillopora damicornis* and *Stylophora pistillata* revealed a small proportion of apparently introgressed hybrids. Again, this can be a case where hybridization is favoured by unusually low densities of conspecific sperm. Recurrent cases of hybridization are certainly favoured by chances of gamete panmixia, such as provided by overlapping spawning events in an aquatic medium. However, coupled to this casuistic explanation there is evidence of adaptive traits being originated or favoured, and that hybridization has a role in range expansion and adaptation to changing environments (Willis *et al.* 2006). Another perspective would be that keeping species barriers relatively permeable would serve the purpose of species with low densities, such as *M. formosa*, or of those with little reproductive success, such as those in marginal or disturbed environments.

Introgression: Genotype vs. phenotype

While introgression can lead to a mosaic composition of the nuclear genome, cytoplasmatic genomes usually experience clonal transmission and are principally maternally inherited, keeping the topologies of more ancestral relationships. This can in part be the case for the mtDNA topology inferred by our analyses. Besides, introgressive evolution is usually not

neutral, meaning that not all genes in the genome are exposed to introgression in the same way. As such, different genes will provide distinct evolutionary interpretations. Eventually, and depending on the genetic region studied, the phenotype may not have a clear relation with the genotype. This can be the case for coral morphologies, which constitute the basis for taxonomic classification in the group (Veron 2000).

Another marker initially included in the study, the nuclear intron *ATPSβ*, did not show variation among *Madracis* species. Although the number of regions included here is limited, contrasting results between markers with respect to shared polymorphism can relate to evidence suggesting that species differentiation is not a genome-wide phenomenon, but has a rather genic basis (Minder *et al.* 2007; Minder and Widmer 2008). Genome-wide approaches support the idea that distinct species sharing hybrid zones have highly porous and weakly differentiated genomes, with the exception of few species-specific markers pointing to the existence of genomic regions sheltered from introgression. In the case of corals, these particular genes could be under the effect of divergent selection, which would prevent introgression in adjoining genomic regions, maintaining phenotypic individuality in hybridizing species despite gene introgression.

Conclusions

We suggest that *Madracis* morphospecies remain recognizable either because hybridization is non-pervasive and/or because disruptive selection is operating. (Marquez *et al.* 2002b; Willis *et al.* 2006). The fact that most species tend to differ in their depth distributions suggests that the species are ecologically differentiated and may be under the effect of disruptive selection. Disruptive selection could work against the homogenising force of gene flow and contribute to the maintenance of morphologically and ecologically distinct species. In this case, depth-related environmental gradients (such as thermoclines) could provide an important template for disruptive selection among coral species. The acclimatisation capabilities of coral symbionts are likely involved in such selective pressures.

Because hybridization may provide options for reef coral resilience under a rapidly changing environment (Willis *et al.* 2006) it should be taken in consideration in conservation strategies. Hybridization events are believed to be widespread on mass-spawning corals, but it was not yet clear whether they have the same prevalence among brooding coral species. This study provides new evidence for frequent occurrence of introgressive hybridization in brooding coral systems.

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