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

L. M. Márquez^{1*†}, M. J. H. van Oppen^{1,2‡}, B. L. Willis² and D. J. Miller¹

¹Department of Biochemistry and Molecular Biology, and ²Department of Marine Biology, James Cook University, Townsville 4811, Australia

High cross-fertilization rates *in vitro* and non-monophyletic patterns in molecular phylogenies challenge the taxonomic status of species in the coral genus *Acropora*. We present data from eight polymorphic allozyme loci that indicate small, but significant, differentiation between sympatric populations of *Acropora cytherea* and *Acropora hyacinthus* ($F_{ST} = 0.025\text{--}0.068$, $p < 0.05$), 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. By contrast, populations of *Acropora 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* ($F_{ST} = 0.427\text{--}0.465$, $p < 0.05$). Moreover, allopatric populations of *A. tenuis* differed significantly, possibly as a consequence of its relatively short period of larval competency. Our results effectively rule out the possibility that *A. hyacinthus* and *A. cytherea* are morphotypes within a single species, and indicate that hybridization occurs relatively infrequently between these taxa in nature.

Keywords: Cnidaria; Scleractinia; interspecific hybridization; introgression; gene flow

1. INTRODUCTION

Most reef-building corals on the Great Barrier Reef (GBR) participate in highly synchronized spawning events (Harrison *et al.* 1984; Willis *et al.* 1985; Babcock *et al.* 1986), which create opportunities for natural hybridization that are unparalleled in the animal kingdom (Babcock 1995) due to high interspecific cross-fertility (Miller & Babcock 1997; Willis *et al.* 1997; Szmant *et al.* 1997; Hatta *et al.* 1999). Genetic studies indicate that introgression—i.e. the interspecific exchange of genes that follows hybridization—may be a common phenomenon in corals. For example, in both *Acropora* and *Madracis*, several species share different types of ribosomal DNA (Odorico & Miller 1997; Diekmann *et al.* 2001). Also, species-level molecular phylogenies of *Acropora*, using both mitochondrial and nuclear markers, show extended para- and polyphyly (Hatta *et al.* 1999; Fukami *et al.* 2000; van Oppen *et al.* 2002). In the mass-spawning genus *Platygyra*, the seven Indo-Pacific species exhibit high rates of interspecific fertilization (Miller & Babcock 1997) and very small genetic distances (Miller & Benzie 1997) indicating that, in this case, hybridization may have led to homogenization of gene pools. Cross-fertilization also occurs among the three members of the *Montastrea annularis* complex (Szmant *et al.* 1997), and there is an ongoing debate about their genetic similarity and taxonomic status (Lopez *et al.* 1999; Medina *et al.* 1999).

Although molecular phylogenetic analyses indicate that interspecific hybridization has played a major role in coral evolution, alternative hypotheses to explain observed patterns of allele and haplotype sharing cannot be rigorously excluded. Two alternative hypotheses that have been widely discussed are (i) that cross-fertile coral species are morphotypes within phenotypically plastic species (Willis *et al.* 1997); and (ii) that these patterns of allele and haplotype sharing reflect recent speciation and retention of ancestral polymorphisms (Diekmann *et al.* 2001; van Oppen *et al.* 2001). Population genetic approaches may have greater potential to distinguish between the rare 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 gain a better understanding of the relationships between cross-fertile *Acropora* species, we compared genetic differentiation between sympatric and allopatric (central GBR versus Western Australian) populations of *Acropora cytherea*, *Acropora hyacinthus* and *Acropora 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* (a mean of 50%) (Willis *et al.* 1997), whereas *A. tenuis* spawns 2–3 hours earlier than its congeners during mass spawning events

* Author for correspondence (lmarquez@jcu.edu.au).

† Present address: Centro de Ecología, Instituto Venezolano de Investigaciones Científicas, Apdo 21827, Caracas-1020, Venezuela.

‡ Present address: Australian Institute of Marine Science PMB No. 3, Townsville, QLD 4810, Australia.

and also has low interspecific compatibility in breeding trials. The comparison of sympatric versus allopatric populations of species with different breeding compatibilities allowed us to establish a meaningful range of genetic distances that correspond with either isolation or interbreeding.

2. MATERIAL AND METHODS

(a) *Sample collection*

Samples were collected from two geographical localities: Trunk Reef (18°24' S, 146°49' E) in the Central Region of the GBR, 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 ca. 500 m²; 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 10 cm pieces from individual colonies. These pieces were maintained in seawater for a maximum of 1 h 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 was confirmed by C. C. Wallace and J. Wolstenholme.

(b) *Electrophoresis*

Tissue extracts were prepared by crushing samples of frozen coral in an equal volume of an extract solution (10 g of sucrose, 0.1 ml of 2-mercaptoethanol per 100 ml of distilled water) (modified from Willis & Ayre 1985). We used a wick impregnated in bromophenol blue for every tenth lane to mark and follow the electrophoretic front. For all species, we initially screened genetic variation at 13 enzyme systems suggested by D. Ayre, and selected eight that gave us a consistently scorable variation: glucose-6-phosphate isomerase (*Gpi*; EC 5.3.1.9) and hexokinase (*Hk*; EC 2.7.1.1) both run on Tris-borate buffer, pH 8.4 (TEB); and malate dehydrogenase (*Mdh*; EC 1.1.1.37), malic enzyme (*Me*; EC 1.1.1.40), phosphoglucosmutase (*Pgm*; EC 5.4.2.2), carboxylesterase (*FL-EST*; EC 3.1.1.1), and two peptidases with leucylglycylglycine and valylleucine substrates (*Lgg* and *Vl*; EC 3.4.11/13) all run on Tris-malate buffer, pH 7.4 (TM7.4). We 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.

(c) *Data analyses*

To test the degree of panmixia within populations, departures of genotypic frequencies from those expected under Hardy-Weinberg (H-W) equilibrium were estimated, using the conventional Monte Carlo Method in TFGA (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 the software GENETIC DATA ANALYSIS (GDA) (Lewis & Zaykin 2000). Values of θ (Weir & Cockerham 1984) were calculated as estimators of

Wright's F_{ST} statistics to determine the degree of differentiation among populations using TFGA (Miller 1997). Probabilities for significant values of $F_{ST} > 0$ were determined by χ^2 -tests across loci and 95% confidence intervals were generated by bootstrapping. A dendrogram, based on Nei's genetic distance (Nei 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.

3. RESULTS

No fixed gene differences were observed among populations of *A. cytherea* and *A. hyacinthus* (see electronic appendix). By 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 1). Significant deviations from H-W equilibrium were noted for some locus-population combinations (table 2). These deviations were mainly due to heterozygote deficits and were correlated with significant inbreeding coefficients (F_{is}) (table 1). 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 the migration of alleles. However, heterozygote deficits that cause deviations from 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 & Hughes 2000). It is unlikely that asexual reproduction is responsible for the heterozygote deficits observed in our study, because we found only one multi-locus genotype repeated once.

The analysis of independence of loci indicated that among 196 pairwise comparisons, 37 were significantly non-independent (table 3). *Me* was the locus that showed the largest number of associations; however, its exclusion did not appear to affect F -statistic estimates significantly (data not shown).

Pairwise Nei's genetic distances and F_{ST} values among populations of *A. cytherea* and *A. hyacinthus* were very small (table 4). Genetic differentiation between sympatric populations of *A. cytherea* and *A. hyacinthus* was significantly larger than that between allopatric populations within each species (table 4; figure 1). *Acropora tenuis* was clearly distinct from the other two species, and significant genetic differences were also observed between allopatric populations of this species. The CatPCA only separated out the two *A. tenuis* populations from the rest with the first principal component explaining 95% of the variation in the data (figure 2). The analysis was not capable of discerning any other groups, even when *A. tenuis* was excluded (not shown).

4. DISCUSSION

(a) *Acropora hyacinthus and Acropora 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 popu-

Table 1. Descriptive statistics for genetic variability in populations of three *Acropora* species.

(* $p < 0.05$, H_o^a : $F_{is} = 0$. D, Dampier, T, Trunk, cyt, *A. cytherea*, hya, *A. hyacinthus*, ten, *A. tenuis*. *A. hyacinthus* and *A. tenuis* are diploid with a somatic chromosome number of 28 (Kenyon 1997). Allozyme patterns suggest that *A. cytherea* has the same ploidy level.)

population	mean sample size per locus	frequency of polymorphic loci	mean no. of alleles per locus	H-W expected heterozygosity	direct count heterozygosity	F_{is}
Dcyt	24.63	1.00	3.50	0.4971	0.4092	0.1799
Dhya	40.13	1.00	3.38	0.4709	0.3314	0.2988*
Tcyt	38.38	1.00	3.38	0.4714	0.3468	0.2670*
Thya	37.38	1.00	3.63	0.5199	0.4017	0.2297*
Wten	43.86	1.00	3.00	0.4410	0.3981	0.0992
Tten	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

^a H_o , observed heterozygosity.

Table 2. D -values [($H_o - H_e$)/ H_e]^a 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 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
Dcyt	-0.1382	-0.5280	0.3342	-0.1203	-0.2564	-0.5812	-0.0891	0.0292
Dhya	-0.5180	-0.6155	0.1133	-0.4236	0.1101	-0.6559	-0.3737	0.0000
Tcyt	-0.1842	-0.4323	0.0975	-0.3989	-0.3555	-0.3992	-0.3452	0.0075
Thya	-0.0871	-0.5372	0.2773	-0.6051	0.0145	-0.3423	-0.2172	-1.0000
Wten	0.1204		0.5157	-0.0439	-0.6491	0.0000	-0.2704	-0.3537
Tten	-0.0377		0.1840	-0.0705	-0.0328	0.2222	-0.2727	-0.2635

^a H_o , observed heterozygosity; H_e , expected heterozygosity.

Table 3. Linkage disequilibrium among loci.

(Each symbol represents a comparison within each of the six populations in the order that 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 4. Pairwise genetic differentiation among populations of three *Acropora* species.

(Nei's (1978) distance below diagonal, F_{ST} above diagonal. * $p < 0.05$, H_o^a : $F_{ST} = 0$. Population codes as in table 1.)

	Dcyt	Dhya	Wten	Tcyt	Thya	Tten
Dcyt		0.025*	0.443*	0.013	0.068*	0.444*
Dhya	0.028		0.454*	0.032	0.047	0.458*
Wten	1.778	1.674		0.452*	0.427*	0.100*
Tcyt	0.015	0.033	1.654		0.067*	0.457*
Thya	0.084	0.052	1.610	0.076		0.430*
Tten	1.981	1.891	0.131	1.884	1.831	

^a H_o , observed heterozygosity.

Figure 1. Dendrogram (UPGMA) of genetic distances (Nei 1978) between sympatric and allopatric populations of *A. cytherea* (open symbols), *A. hyacinthus* (grey symbols) and *A. tenuis* (black symbols) in the GBR (circles) and Western Australia (stars).

Figure 2. Ordination produced by the CatPCA of genotypic data of sympatric and allopatric populations of *A. cytherea* (c), *A. hyacinthus* (h), and *A. tenuis* (t) in the GBR (g) and Western Australia (w).

lations from distant geographical locations (i.e. eastern versus western Australia) could not be distinguished. These results indicate that hybridization occurs at a very low frequency, i.e. introgression is lower than intraspecific gene flow between widely separated populations; therefore the species are unlikely to be merging. The same pattern was observed when a comparable published allozyme dataset (Ayre & Hughes 2000) was reanalysed (figure 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, we 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 dataset were larger than those obtained in our study, probably because Ayre & Hughes

Figure 3. Dendrogram (UPGMA) of genetic distances (Nei 1978) between populations of *A. cytherea* and *A. hyacinthus* in the GBR, reanalysed from Ayre & Hughes (2000). Lizard Island (triangles); Central GBR (circles); Heron Island (squares); *A. cytherea* (open symbols); *A. hyacinthus* (grey symbols).

only used four highly polymorphic loci and because our scoring was more conservative. Unfortunately, these differences in scoring methods made it impossible to combine our datasets.

Our 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 & Benzie 1997), mass-spawning coral species that are also known to hybridize efficiently *in vitro* (Miller & 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.

(b) *Incipient species or secondary contact?*

There is no fossil record for either *A. cytherea* or *A. hyacinthus* prior to the Pleistocene era (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 (Márquez *et al.* 2002); 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.

Among Indo-Pacific species of *Acropora*, temporal differences in spawning time appear to be the major determinant of genetic distinctness (van Oppen *et al.* 2001, 2002), with simultaneously spawning species generally being poly- or paraphyletic. *Acropora 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 the extremely low genetic differentiation. In summary, we 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.

(c) Larval competence and allopatric genetic differences within species

The genetic differentiation between eastern and western 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 geographical 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 (less than 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, personal communication). 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).

5. 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 (Márquez *et al.* 2002). 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 & 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. Alternatively, it has been suggested that disruptive selection could maintain ecologically differentiated coral morphotypes (Willis *et al.* 1997), while a certain level of assortative mating can maintain these morphotypes genetically distinct (Dieckmann & 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.

We thank J. McKenzie, J. Cruz and various volunteers for their help collecting the samples. We gratefully acknowledge the technical assistance of B. Ballment, C. Bastidas, S. Uthicke and D. Ayre, L. Smith and A. J. Heyward for assistance during our fieldwork in Dampier, and C.C. Wallace and J. Wolsten-

holme for identification of samples. We also thank the Australian Institute of Marine Science (AIMS) for access to the Dampier Research Station and laboratory facilities at Cape Ferguson. This project was funded by grants from the Australian Research Council and James Cook University. L.M.M. acknowledges receipt of a PhD scholarship from the Venezuelan Council for Scientific Research.

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