RESEARCH ARTICLE

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Temporal reproductive isolation and gametic compatibility are evolutionary mechanisms in the *Acropora humilis* species group (Cnidaria; Scleractinia)

Abstract Patterns of interbreeding between individuals are fundamental to the structure and maintenance of evolutionary boundaries between species. In corals, both hybridisation and reproductive isolation appear to be important evolutionary mechanisms. In this study, I examine evolutionary boundaries using morphological, molecular and reproductive criteria within the *Acropora humilis* species group at Lizard Island on the Great Barrier Reef, Australia. Five species and seven morphs are recognised on the basis of morphological appearance of features traditionally used to identify corals of the genus *Acropora*. In a molecular phylogenetic analysis, I examine relationships for the mitochondrial DNA's putative control region, using maximum-parsimony and maximum-likelihood methods. The reproductive criteria explore whether species and morphs are reproductively isolated on the basis of temporal or fertilisation barriers. Timing of gamete maturity is surveyed for each species and morph, from the month prior to and 3 months after the mass spawning. Time of spawning is documented at the levels of night and hour of spawning, and time taken for egg-sperm bundles to separate. Laboratory fertilisation experiments tested the potential of species and morphs to interbreed. High levels of intraspecific and extremely low or zero fertilisation levels between the five species indicated that they are valid species. Based on the combined assessment of morphological, molecular and reproductive criteria, *A. humilis* and *A. gemmifera* appear to be the most closely related species, which are most closely related to the remaining species in the following order: *A. samoensis*, *A. monticulosa* and *A. digitifera*. Evidence derived from one or more of these criteria suggest that the morphs (1) are at various stages of divergence from the species with which they share morphological characters, and (2) may indicate possible zones of speciation and hybridisation. Identification of morphs avoided the possibility of taxonomic error and was essential for accurate interpretation of evolutionary boundaries. Confirmation of morphology as an informative character of evolutionary boundaries is of great significance because most coral research projects rely on morphology as the primary tool for identification of species.

Keywords *Acropora* · Morphology · mtDNA · Reproductive isolation · Species boundaries

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Introduction

Hybridisation is an important mechanism of speciation in many groups of plants and animals (reviewed in Arnold 1997; Rieseberg 1997). Hybridisation may also promote speciation in scleractinian corals (Willis et al. 1997) and may contribute to the taxonomic difficulties of defining boundaries between species (Wallace and Willis 1994; Babcock 1995; Veron 1995). However the extent to which hybridisation occurs in nature, its evolutionary role and its phenotypic effect in corals are unknown.

Many corals reproduce during synchronous multi-specific spawning events (Harrison et al. 1984; Babcock et al. 1986, 1994; Hayashibara et al. 1993), potentially providing opportunities for interspecific hybridisation (Babcock 1995; Willis et al. 1997). Gametes are viable for 6–8 h after spawning (Willis et al. 1997) and those released from species that spawn synchronously aggregate and mix at the water surface, providing the potential for fertilisation between different species. Interspecific fertilisation occurs under laboratory conditions (Knowlton et al. 1997; Miller and Babcock 1997; Szmant et al. 1997; Willis et al. 1997; Hatta et al. 1999; Fukami et al. 2003) and some molecular studies conclude that common DNA sequence types in different species of

corals are evidence of interspecific hybridisation (Odorico and Miller 1997; Hatta et al. 1999; van Oppen et al. 2000, 2001, 2002b; Diekmann et al. 2001; Fukami et al. 2003). A study of chromosome numbers concluded that hybridisation contributed to the development of polyploidy and rapid speciation in the genus *Acropora* (Kenyon 1997).

Although there is potential for hybridisation in corals, prezygotic mechanisms also appear to be important in limiting interspecific breeding in corals. The most apparent prezygotic mechanisms in corals include temporal reproductive isolation and gametic incompatibility. Temporal isolation has been proposed for some species on the scale of hours within mass-spawning periods (Knowlton et al. 1997; Szmant et al. 1997; van Oppen et al. 2001; Fukami et al. 2003). Many additional species spawn up to 1–3 h apart (Babcock et al. 1986; Wallace 1999) and may also be reproductively isolated. Other species spawn weeks or months out of phase with the mass spawning (Willis et al. 1985; Babcock et al. 1986; Hayashibara et al. 1993; Wallace 1999; Hayashibara and Shimoike 2002) and may be partially or completely reproductively isolated by temporal barriers. Gametes of many species also appear to be incompatible. Within synchronously spawning species of *Acropora*, rates of intraspecific fertilisation in experimental crosses are high (often >90%) for many species compared with rates of fertilisation in many interspecific crosses (Figs. 3a, 4a in Willis et al. 1997; Table 1 in Hatta et al. 1999). The likelihood of interspecific fertilisation in coral spawning slicks also appears to be reduced by the presence of sperm attractants in eggs, which enhance conspecific fertilisation and reduce interspecific fertilisation, as demonstrated for species of *Montipora* (Coll et al. 1994).

Detailed examination of morphologically similar species and intraspecific morphs, using molecular and reproductive criteria, suggest that evolutionary boundaries within and between coral taxa are at various stages of formation. In one study, congruent patterns of shared DNA sequences and high levels of fertilisation between morphologically divergent species of *Acropora* provide strong evidence of interspecific hybridisation (Hatta et al. 1999). In the same study, Hatta and colleagues also recognised three morphs in *A. nasuta* and two in *A. muricata* (synonym of *A. formosa*), which showed high levels of intramorph fertilisation and (in all but one cross) extremely low levels (≤ 1.6%) of intermorph fertilisation. This suggests that breeding boundaries have formed between the morphs within each of these species. Márquez and colleagues demonstrate that the species *A. hyacinthus* and *A. cytherea* are closely related but have now evolved to form statistically distinct lineages which hybridise infrequently in nature, despite having high levels of hybridisation in laboratory experiments (Márquez et al. 2002a, b). Two studies have concluded that *Acropora palmata* and *A. cervicornis* are evolutionarily distinct species and that *A. prolifera* is a hybrid, derived from these species (van Oppen et al. 2000; Vollmer and Palumbi 2002). The latter study also demonstrated that colonies of *A. prolifera* are first generation hybrids with limited potential to interbreed, concluding that the effect of hybridisation has been the generation of new morphologies without speciation. Three species of *Montastraea*, initially described as separate species and then synonymised within *Montastraea annularis*, are now recognised on the basis of morphological, molecular and behavioural differences, as well as timing of spawning and fertilisation potential (Knowlton et al. 1997; Szmant et al. 1997). However, no single character has been found which separates the three species. This and the variable occurrence of morphologically intermediate colonies suggest that boundaries may be at different stages of formation in different locations within the Caribbean (Knowlton et al. 1997; Szmant et al. 1997). Similarly, the species pairs *A. millepora* and *A. spathulata* and *Montipora digitata* and *M. tortuosa* were each previously regarded as single morphologically variable species, but are now recognised as distinct species on the basis of morphological and breeding criteria (Wallace 1999; Stobart 2000), and fixed genetic differences for the *Montipora* species (Stobart and Benzie 1994). In the genus *Platygyra*, seven morphospecies from the Great Barrier Reef have been defined in multivariate analyses, although no single morphological, reproductive or genetic character has been found that separates them (Miller and Babcock 1997; Miller and Benzie 1997). As suggested by Willis et al. (1997), based on the small number of species in the genus *Platygyra* compared with the genus *Acropora*, the effect of hybridisation may have been to merge species or retard speciation in the former genus, but promote speciation in the latter genus.

These studies clearly indicate that it is necessary to use morphs, rather than species, as sampling units to interpret evolutionary boundaries in corals accurately. This will provide the greatest opportunity for elucidating evolutionary relationships in corals, separating genetic versus environmental influences on morphological variability, and avoiding the potential of confused phylogenies due to taxonomic error. In this study, I examine evolutionary relationships between morphs of the *A. humilis* species group at Lizard Island (Great Barrier Reef), defined on the basis of morphological appearance. The major aim of this project is to determine the extent to which morphology is indicative of evolutionary relationships within and between currently defined species in this group of corals. To do this, I use molecular and breeding criteria. The *A. humilis* species group is of particular interest because species within this group have a high level of intraspecific morphological variability, with boundaries between species appearing indistinct (Wallace 1999). In addition, based on previous surveys at Lizard Island (Wallace 1999; B. Kojis personal communication), it appears that temporal reproductive isolation may occur within this species group.

Materials and methods

Species and morphs

The sampling units used in this study were putative morphs, distinguished using the morphological characters that are traditionally used to identify species of the genus *Acropora* (Wallace 1999; Wolstenholme et al. 2003). These putative morphs were defined during pilot searches and include known species, and morphs within and between these species. Morphs are named using abbreviations from the species with which they share most morphological similarity. Characters used to distinguish the species and morphs are summarised in Table 1.

Relative abundance

Surveys were carried out to assess the relative abundance of each putative morph in the *A. humilis* species group at Lizard Island (14° 40′ S, 145° 28′ E). Surveys were conducted in the five habitats in which colonies of the *A. humilis* species group commonly occur at Lizard Island. These habitats were exposed reef flats, exposed crests, exposed slopes, lagoonal margins of the reef flat and lagoonal patch reefs. Surveys were conducted at two locations for each habitat. The two locations of the exposed habitats were approximately 6 km apart, facing north-easterly and southerly directions. Lagoonal reef flat locations were approximately 1 km apart at the south-east of the Lizard Island lagoon, and lagoonal patch reefs were approximately 0.5 km apart at the north-west of the lagoon. All habitats except the slope were shallow, ranging in depth from about 0 to 2 m. The slope habitat ranged in depth from about 4 m, at the edge of the reef wall to about 15–20 m, where corals were extremely sparse or absent.

In each survey, the first hundred colonies encountered from the *A. humilis* species group were identified as one of the putative morphs. Surveys were conducted over a distance of approximately 50–200 m in the crest, flat and patch reef habitats. Colonies of the *A. humilis* species group were more sparsely distributed in the slope habitats and therefore it was necessary to search distances of 1–2 km. Five replicate surveys were made for each habitat, except the lagoon patch reefs for which only three replicate surveys were possible due to the small size of the reefs. Colonies that were too small to be confidently identified (usually < 5 cm) were not included. Due to the very high abundance of colonies of the *A. humilis* group on the reef crests at Lizard Island, surveys were conducted along a 1-metre belt transect to ensure less conspicuous colonies were included. Surveys in the other habitats were conducted by haphazard swimming, with all colonies encountered being identified.

Molecular techniques and analysis

Molecular samples were collected to analyse genetic relationships between the putative morphs. One or two colonies of all but two of the rarer morphs ("mont-hum" and "mont-gem") and three colonies of *A. austera*, the outgroup taxon for this study, were sampled. The species *A. austera* was selected as the most appropriate outgroup because it is directly ancestral to the species examined in this study, based on a morphological phylogeny of the genus *Acropora* (Wallace 1999). For each colony sampled, skeletal branch samples were collected and photographs taken, providing a reference of the appearance of the colony. Molecular samples were collected following the protocol described by Wolstenholme et al. (2003).

DNA was extracted from a volume of approximately 200 μl tissue and skeleton, using a Viogene blood and tissue genomic DNA extraction kit. The tissue and skeleton were ground to a fine powder in liquid nitrogen, mixed with an equal volume of lysis buffer and 20 μl proteinase E. Samples were incubated overnight in a 60°C water bath. DNA was extracted as per the manufacturer's instructions and precipitated in isopropanol. Following precipitation, the genomic DNA was dried, resuspended in double-distilled H_2O and stored at −20°C. The marker used for the molecular analysis was the mtDNA putative control region [referred to as the mtDNA intergenic region for the remainder of this paper, as in van Oppen et al. (2001)]. Target segments were amplified using the primers AcrdloopF (5′-TGTTAGTACAAATCGCCCGTCGCC-3′), AcrdloopInt(5′-CGTGAGCAGGACGCTTCAG-3′) and AcrdloopR (5′-CATCCATATCATTTGGTTGAGCCTTCT-3′), designed by van Oppen et al. (1999). The amplification reaction used 100–200 ng DNA template and BRL *Taq* polymerase in a 50 μl reaction, in the presence of the buffer supplied with the enzyme (as per manufacturer's instructions). PCR was performed in a PC-960G gradient thermal cycler using the following steps: incubation period of 4 min at 95°C; 5 cycles of 30 s at 94°C, 1 min at 50°C and 2 min at 72°C; followed by 30 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C; ending with a 10 min extension at 72°C. PCR products were subjected to electrophoresis in a 0.8% agarose (FMC Bioproduct) gel in 1×TAE (Tris-acetate-EDTA) buffer to assess the yield.

Sequences were initially obtained by direct sequencing but these could not be interpreted, apparently due to the variable occurrence of repeat-sequence blocks within individuals. All sequences analysed in this study were therefore obtained by cloning. Multiple clones were sequenced from most individuals (Table 2) enabling variation within and between individuals to be compared. PCR products were cloned using the ligation kit, pGEM T easy (Promega) and transformed into DH5α competent cells (BRL), under conditions recommended by the manufacturers. Bacterial colonies containing the vector were picked with a sterile toothpick and cultured for 6–12 h in a 4 ml LB (Luria-Bertani) nutrient solution and purified using a Viogene plasmid DNA mini-preparation kit. Nucleotide sequences were generated for both strands on an ABI 377 genetic analyser using the ABI Big-dye ready reaction kit following standard cycle-sequencing protocol.

Sequences were aligned manually in Seqapp 1.99 (Gilbert 1994). Calculation of the pair-wise sequence distance matrix and the maximum-parsimony (MP) and maximum-likelihood (ML) phylogenetic analyses were performed in PAUP* 4.0b10 (Swofford 2002). Phylogenetic analyses used the heuristic search option. Bootstrapping with 1,000 pseudoreplicates determined the robustness of clades, with branches supported by < 50% being collapsed as polytomies. The MP analysis was run with gaps excluded from the analysis, as well as treating gaps as a fifth character. The best-fit model of sequence evolution (TrN + I) was determined using Modeltest 3.06 (Posada and Crandall 1998) and the Akaike information criterion (AIC) method, for the ML analysis.

Sequences have been submitted to GenBank under accession numbers AY364090 to AY364162 and the morphological reference samples are deposited at the Museum of Tropical Queensland (MTQ), Townsville, Australia (registration numbers G56366–G56383). GenBank accession numbers and MTQ registration numbers are cross-referenced with the reciprocal institutions.

Breeding potential

Timing of gamete maturity

Surveys of the six most abundant morphs were carried out from November 1998 to December 2001, to determine the proportion of colonies containing mature eggs and an indication of the timing of spawning of each morph. Surveys were conducted 1–3 days after the full moons during the spawning season, which extended from 1 month before and 3 months after the mass-spawning event (months −1, 0, 1, 2, and 3 where month 0 is the mass-spawning month). Up to 30 colonies of *A. humilis*, *A. gemmifera*, *A. samoensis*, *A. digitifera*, *A. monticulosa* and the morph "dig-gem" were surveyed each month, with fewer colonies surveyed during the earlier spawning seasons. Surveys were conducted around Lizard Island for all morphs. Colonies of *A. digitifera* are rare at Lizard

Table 1 Primary characters used to identify species and morphs in the *Acropora humilis* species group

Species	Growth form	Branch shape	Axial corallites	Radial corallites[a]	Colony colour
A. samoensis	**Caespito-corymbose**	Thin, terete	Large	One size, large, tubular to tubo-nariform, **not crowded**	Brown with edges of radial corallites, calice of axial corallites and ring around axial corallites pale green-yellow, pale brown, white or pale blue; polyps green-yellow (in colonies with this colour), white or pale brown
A. humilis	Corymbose	Thick, terete	**Large to very large**	**One size**, large, **nariform to tubo-nariform**, crowded	Often brightly coloured: brown, green, blue, purple or combinations of these colours; often paler or white around axial corallites; polyps yellow, green, white, pale blue or pale brown
A. gemmifera	Corymbose	Thick, conical	Large	**Two sizes**, large, **flaring dimidiate to tubo-nariform**, crowded	Brown or pale brown, often with an orange, blue or yellow tinge; branch tips, edges of radial corallites and edges of colony often paler or white; polyps white or pale brown
A. monticulosa	**Digitate**	Very thick, **very conical**	Small	One size, small, **nariform**, crowded	Colonies usually the same colour along length of all branches; brown with pale or strong green-yellow or purple-blue tinge; polyps green-yellow (in colonies with this colour), white or pale brown
A. digitifera	Corymbose	Very thin, terete	Small	Mixed sizes, large, **flaring dimidiate to flaring lips**, crowded	Pale brown to brown; sometimes with blue or cream branch tips; polyps pale or dark brown
Morphs[b]					
"Sam-hum"	Corymbose to caespito-corymbose	Thin, terete	Large	**One size**, large, **tubo-nariform**, usually crowded	Usually as described for *A. samoensis*, but sometimes a single colour within the range of that seen in *A. humilis*
"Sam-gem"	Corymbose to caespito-corymbose	Thin, terete	Large	**One size**, large, **tubo-nariform to dimidiate**, usually crowded	Same as *A. samoensis*
"Hum-gem"	Corymbose	Thick, terete	Large	One to two sizes, large, **tubo-nariform to dimidiate**, crowded	Colonies similar in colour to *A. humilis* but not usually as brightly coloured; polyps white or pale brown
"Mont-hum"	Corymbose	Thick, terete to conical	**Large to very large**	**One size**, large, **nariform**, crowded	Colonies recorded in this study were usually brown sometimes with a pink or yellow tinge; often calices of radial and axial corallites and ring around axial corallite were paler or white; polyps white
"Mont-gem"	Digitate	Very thick, **very conical**	**Small**	**Two sizes**, small, **dimidiate**, crowded	Same as *A. gemmifera*
"Terete mont"	Digitate	Very thick, **sometimes conical**	Large	Mixed sizes, small, dimidiate, crowded	Brown with a green-yellow or purple-blue tinge, calices of radial and axial corallites paler in colour, polyps white
"Dig-gem"	Corymbose	**Thick, conical**	Small	Mixed sizes, large, **flaring dimidiate to flaring lips**, crowded	Same as *A. digitifera*

[a] The first size category in the description of radial corallites refers to the size range of radial corallites within that morph and the second size category refers to the size of radial corallites relative to other morphs

[b] Morphs are named according to the species with which they share most morphological characters; characters that are most important for distinguishing each species and morph are highlighted in bold font

Table 2 Number of sequences obtained from individuals of each species and morph of the *A. humilis* species group in the molecular analysis. GenBank Accession numbers and corresponding Museum of Tropical Queensland (MTQ) registration numbers for morphological reference samples are listed for each colony

Species or morph	Sample code	Number of sequences per Individual	Number of base differences between sequences within an individual	GenBank accession numbers[a]	MTQ registration number
A. samoensis	119	5	1–7	AY364090–4	G56366
	121	5	2–4	AY364095–9	G56367
A. humilis	23	2	2	AY364100–1	G56368
	32	3	2–7	AY364102–4	G56369
A. gemmifera	108	4	0–10	AY364105–8[a]	G56370
A. monticulosa	30	8	0–9	AY364109–16[a]	G56371
	34	4	3–6	AY364117–20	G56372
A. digitifera	122	1		AY364121	G56373
	124	5	0–3	AY364122–6	G56374
"Sam-hum"	94	5	1–3	AY364127–31	G56375
"Sam-gem"	95	3	0–2	AY364132–4	G56376
"Hum-gem"	105	5	1–7	AY364135–9	G56377
"Terete mont"	6	1		AY364155[a]	G56378
"Dig-gem"	27	9	1–6	AY364140–8	G56379
	35	6	2–10	AY364149–54	G56380
A. austera	111	3	2–6	AY364156–8[a]	G56381
	112	2	5	AY364159–60[a]	G56382
	113	2	9	AY364161–2[a]	G56383

[a]Sequences with the accession numbers AY364106 (*A. gemmifera*); AY364111, AY364112 and AY364116 (*A. monticulosa*); AY364155 ("terete mont"); and all sequences from colonies of *A. austera* did not contain the repeat sequence block, as described in the text

Island (Fig. 1) but common at a nearby island, North Direction Island (5 km south-east of the Lizard Island group). Surveys of 30 colonies were therefore conducted at North Direction Island for *A. digitifera*, with fewer colonies sampled around Lizard Island during the earlier phases of the project. Based on the results of these surveys, no difference in timing of spawning was evident between Lizard and North Direction Islands.

The surveys were conducted following the protocol described in Baird et al. (2002). Eggs develop over a period of approximately 9 months in species of *Acropora*, becoming pigmented about 3 weeks prior to spawning (Wallace 1985). Testes are visible microscopically 4–6 weeks prior to spawning (Wallace 1985). To determine timing of gamete maturity and month of spawning, branches were broken from colonies and scored as follows: colonies in which eggs were not visible or only just visible microscopically were scored as containing "no eggs"; colonies with visible but white eggs were recorded as "immature"; and colonies with pigmented eggs were recorded as "mature" and ready to spawn. Up to five branches were broken from each colony. If any of these branches contained mature eggs, then the colony was scored as mature. If reproductive status could not be confidently assessed in the field (i.e. "no eggs" vs "immature"), branch samples were collected, preserved in 5% formalin, decalcified in 3% hydrochloric acid and examined under a dissecting microscope.

Spawning and fertilisation trials

Laboratory-based fertilisation experiments were carried out, following the procedure in Willis et al. (1997). Fertilisation trials were only conducted between colonies spawning during the same period of gametic viability (i.e. the same night), to test the breeding potential within and between species and morphs that could feasibly interbreed in nature. Potential for colonies to interbreed was tested between pairs of colonies of the same morph, between pairs of colonies of different morphs and gametes from a single colony (selfs) for all morphs, except the rare morph "mont-gem".

Colonies used in the fertilisation experiments were collected 1–5 days after the full moon. Each colony was placed in a separate aquarium. Once colonies had released a substantial amount of spawn, egg-sperm bundles were collected and poured into a cup with a plankton mesh base, in a bowl of seawater. The cup was then gently agitated to separate egg-sperm bundles, with sperm being strained through the plankton mesh and eggs retained within the cup. Plankton mesh with a pore size of 210 μm was used in the main spawning month. However, in the first spawning season, I found that this mesh was too coarse to retain eggs released from colonies that spawned in the later months (months 2 and 3). Plankton mesh with a pore size of 62 μm was therefore used to separate eggs and sperm in the later months. Sperm diluted to approximately 10^6 per ml and approximately 100 eggs were then combined in 25-ml glass vials. Controls were set up for each colony used in the experiment, to ensure that extraneous sperm had not contaminated any of the crosses. All crosses and controls were replicated three times. Vials were suspended at the surface in a tank of aerated water, simulating the conditions in which eggs and sperm are mixed in a spawning slick. The proportions of regular embryos were counted after 6–10 h. Fertilisation levels in all self crosses were <10% and usually <3%, indicating that selfing is possible but most probably a consequence of the artificial conditions. Fertilisation levels in all controls were <3% and usually 0%. Recorded fertilisation levels may therefore include occasional embryos due to selfing or sperm contamination. Crosses that showed low levels of fertilisation were therefore interpreted cautiously. Time of spawning (nights after the full moon and time of night) and time taken for gametes to separate were also recorded, providing additional indications of breeding potential within and between morphs.

Results

Species and morphs

Twelve morphs were recognised within the *Acropora humilis* species group. Five of the morphs corresponded with currently recognised valid species in this group and are referred to as species for the rest of this paper,

Fig. 1a–f Relative abundance of species and morphs in the *Acropora humilis* species group at Lizard Island in five habitats. **a** Overall abundance for all habitats. **b–f** Abundance in each habitat. *Columns* represent average number of colonies for five replicate surveys for crest, flat and slope habitats and three replicate surveys for lagoon patch reefs. *Black* and *white bars* distinguish the two sites surveyed for each habitat. *Error bars* indicate 1 SD. Species and morphs are ordered along the horizontal axis by overall abundance (most to least abundant) in non-slope and then slope habitats. *Upper case letters* represent species and *lower case letters* represent morphs as follows: *dg* "dig-gem", *G A. gemmifera, hg* "hum-gem", *H A. humilis, M A. monticulosa, tm* "terete mont", *mh* "mont-hum", *mg* "mont-gem", *D A. digitifera, S A. samoensis, sh* "sam-hum", *sg* "sam-gem"
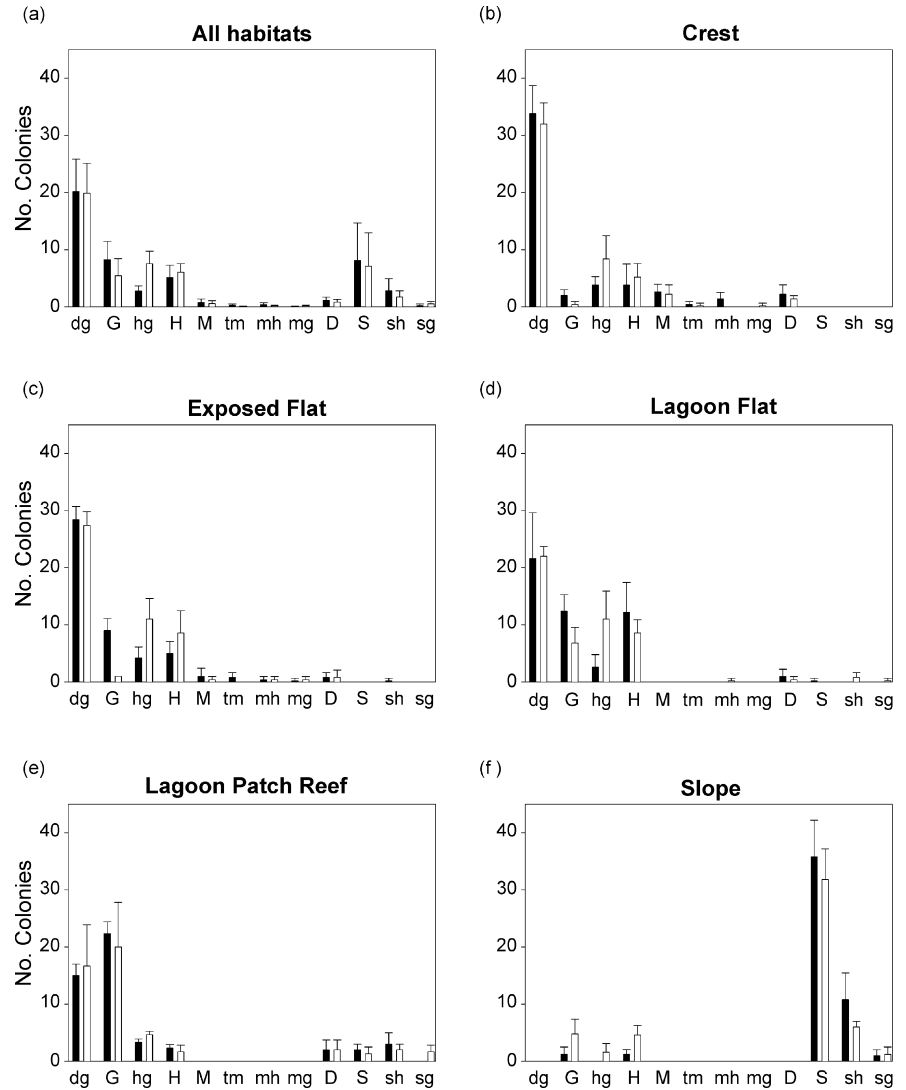
distinguishing them from the other seven morphs. The species are *A. humilis* (Dana 1846), *A. gemmifera* (Brook 1892), *A. samoensis* (Brook 1891), *A. monticulosa* (Brüggemann 1879) and *A. digitifera* (Dana 1846). Six of the remaining seven morphs were morphologically intermediate between these species and are named after the species with which they appear to share greatest morphological affinity (Table 1). The seventh morph, "terete mont" appeared to be most closely associated with *A. monticulosa*, and is named for its apparent affinity with this species and its less conical (terete) branches. Four of the morphs, "dig-gem", "mont-hum", "mont-gem" and "terete mont" were distinct. The "hum-gem" morph was morphologically variable, forming a continuum between the species *A. humilis* and *A. gemmifera*, with some colonies appearing most similar to one of these species and other colonies clearly sharing characters with both species. Colonies of the morphs "sam-hum" and "sam-gem" each appeared to share closest morphological affinity with *A. samoensis*.

Relative abundance

The most abundant species or morph was the morph "dig-gem", dominating the crest and flat habitats, common on the lagoon patch reefs but absent on the slopes (Fig. 1). *A. humilis*, *A. gemmifera* and the morph "hum-gem" were moderately common in all habitats. Colonies of *A. monticulosa* and morphs sharing characters with this species were mainly found on the reef crest, but were not abundant in any habitat and were absent on the lagoon patch reefs and slopes. *A. digitifera* was present but rare at all shallow sites and absent on the slopes. *A. samoensis* was the most abundant species or morph of the *A. humilis* species group in the deeper slope habitat, with "sam-hum" being the next most abundant. Both of these morphs were extremely rare or absent in all exposed habitats, while a small number of colonies were present on the lagoon patch reefs. The morph "sam-gem" was always rare, with only a few colonies being recorded on the slope and one lagoon patch reef.

Molecular phylogenetic analysis

Phylogenetic analysis of the mtDNA intergenic region, using both MP and ML methods, divided the species and morphs into two clades. *A. digitifera* and "dig-gem" formed clade I and all other species and morphs formed clade II (Fig. 2). Strong (100%) bootstrap support for clade I indicates that *A. digitifera* and "dig-gem" are distinct from the other species and morphs of the *A. humilis* species group for this marker. Within clade I, sequences from the colonies of *A. digitifera* and "dig-gem" were indistinguishable. Within clade II, divergence is also apparent for *A. monticulosa* (clade IIa) from *A. humilis*, *A. samoensis*, *A. gemmifera* and intermediate morphs of these three species (clade IIb), with each of these subclades having strong (95%) bootstrap support. There was little additional resolution in clade IIb, suggesting a close evolutionary relationship between these species and morphs.

Repeat-sequence blocks were present in some but not all cloned sequences from single colonies of *A. gemmifera*, *A. monticulosa*, "terete mont" and all cloned sequences from the three colonies of *A. austera* (Table 2), indicating that these repeat sequences do not represent a consistent phylogenetic signal. The repeat-sequence blocks were therefore deleted prior to the phylogenetic analysis. In addition to the repeat-sequence blocks, there were low levels of variation between sequences cloned from each individual. The number of base differences between cloned sequences from an individual coral ranged from 0 to 10 (Table 2). This variation may indicate polymorphic sites within an individual or PCR error. Single consensus sequences for each individual were therefore used in the analyses. At nucleotide sites that varied within an individual, the most commonly occurring base at that site was used in the consensus sequence. If different bases occurred with equal frequency at a particular site, that site was recoded using the IUPAC (International Union of Pure and Applied Chemistry) ambiguity code.

Base composition was homogeneous between sequences from morphs of the *A. humilis* species group and *A. austera* (Table 3). The level of divergence between sequences obtained in this study was low (Table 4), as also reported in Márquez et al. (2002b) and van Oppen et al. (2001) for species of the genus *Acropora*. Maximum sequence divergence between species and morphs of the *A. humilis* species group and *A. austera* was 6.4% (Table 4). This was similar to the level of divergence for a broad range of species of *Acropora* (6.9%) reported in

Fig. 2 Maximum-parsimony consensus tree (50% majority rule) with mid-point rooting, produced in the analysis of the mtDNA intergenic region for species and morphs of the *A. humilis* species group. Gaps were treated as missing characters in this analysis. *Numbers below branches* indicate bootstrap values (1,000 replicates) for branches with >50% support. *Species names* and *sample codes* are given for each individual. *Sample codes* are as listed in Table 2
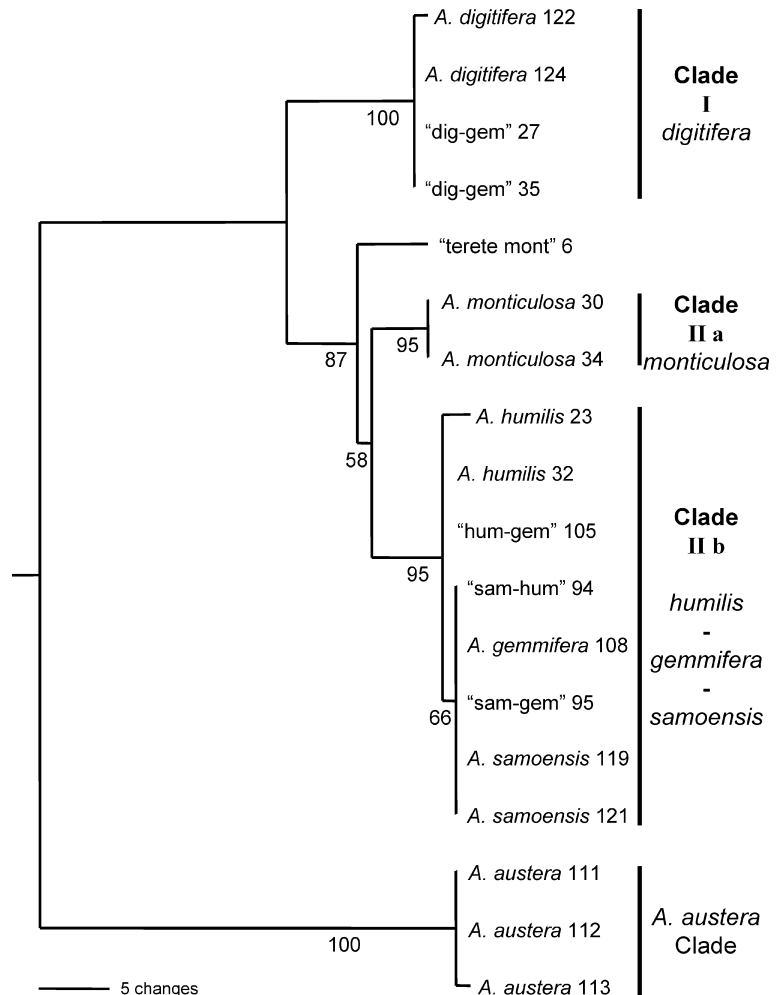
Table 3 Mean base compositions (%) for species and morphs of the *A. humilis* species group and *A. austera*. Standard deviations are given in parentheses

A	C	G	T
24.7 (0.002)	17.1 (0.002)	26.4 (0.002)	31.7 (0.004)

Table 4 Average Kimura 2-parameter pair-wise sequence distances (%) within and between species and morphs of the *A. humilis* species group and *A. austera*. Maximum values are given in parentheses

	Within morphs	Between morphs	*A. austera*
A. humilis species group	0.7 (1.3)	1.4 (3.1)	5.6 (6.4)
A. austera			0.5 (0.8)

van Oppen et al. (2001) and double the level of divergence between sequences from species and morphs within the *A. humilis* species group. Despite this distinction, sequences from colonies of *A. austera* were not sufficiently different to form a natural outgroup. Trees produced in the phylogenetic analyses were therefore constructed using the midpoint rooting option. The aligned consensus sequences consisted of 1,075 positions with repeat-sequence blocks deleted. Prior to deletion of repeat-sequence blocks, individual sequences ranged in length from 1,094 to 1,233 bp. Within the aligned sequences, 999 positions were constant, 7 variable characters were not parsimony-informative and 69 were parsimony-informative.

MP and ML analyses produced trees with similar topologies and levels of bootstrap support, differing in that the single "terete mont" sequence formed a third branch with weak bootstrap support in clade II in the MP analysis (Fig. 2) and a polytomy within this clade in the ML analysis. Additional sequences are needed to clarify the phylogenetic position of this morph, but based on the analyses in this study, it appears to be distinct from the species *A. monticulosa*. Treating gaps as a fifth character in the MP analysis did not change the tree topology, as also reported by van Oppen et al. (2001). MP analysis with gaps treated as missing characters produced 607 most parsimonious trees of 81 steps. A consistency index of 0.938, homoplasy index of 0.062 and a retention index of 0.973 indicate a strong phylogenetic signal in the sequence data.

Breeding potential

Reproductive barriers

The results of this component of the study demonstrate that prezygotic mechanisms restrict the interbreeding potential between species in the *A. humilis* species group. Temporal reproductive barriers were present at two levels, i.e. month of gamete maturity and hour of spawning, while isolation of species or morphs was not evident on the basis of day of spawning. Fertilisation barriers also existed between species that spawned synchronously (i.e. on the same night, within a 4 h period).

Timing of gamete maturity

Surveys of the timing of egg maturity in colonies of the *A. humilis* species group at Lizard Island provided a direct indication of timing of spawning for each morph, for each month of the spawning season (Fig. 3). The development of eggs through the three stages of maturation is evident for each morph (Fig. 3) and timing of spawning was validated by field and laboratory observations. No spawning was recorded in the days prior to collection of this survey data. Spawning was only recorded by morphs containing mature eggs, and colonies recorded with mature eggs in the surveys contained no eggs in the days following spawning (Table 5).

The most striking finding in the pattern of timing of gamete maturity was that a second substantial spawning event occurred 3 months after the mass spawning, with all but one species spawning only during one of these events (Fig. 3a). Colonies of *A. monticulosa* had the greatest level of synchronicity, with almost all colonies (96%) containing mature eggs during the mass-spawning month (month 0) and 4% during the month following the mass spawning (month 1). Similarly, most colonies of *A. humilis* and "dig-gem" contained mature eggs during the mass-spawning month (90% and 89% respectively). In contrast, *A. samoensis* never contained mature gametes during the mass spawning, with all colonies of this species spawning during months 1, 2 or 3. The absence of mature gametes in colonies of *A. digitifera* during month 0 indicates that it also is not a mass-spawning species. The predominant time of spawning for this species appears to be month 3, based on the data available, with almost all colonies (94%) containing mature eggs in this month. *A. gemmifera* showed the greatest variability in timing of spawning, with high proportions of colonies containing mature eggs during the mass spawning and 3 months afterwards (55% and 32% of all colonies respectively), with ≤ 8% of all colonies spawning in each of the other months of the spawning season.

Colonies of the rarer morphs were also scored when encountered during the survey period. Colonies of "mont-hum", "mont-gem" and "terete mont" were only recorded with mature eggs in month 0. The majority of colonies of "hum-gem" also contained mature eggs during month 0 (83%), with ≤ 7% of colonies containing mature eggs in each of the other months surveyed. Colonies of "sam-hum" and "sam-gem" appear to follow the timing of spawning recorded for *A. samoensis*, with sampled colonies containing mature eggs only during months 2 and 3.

575
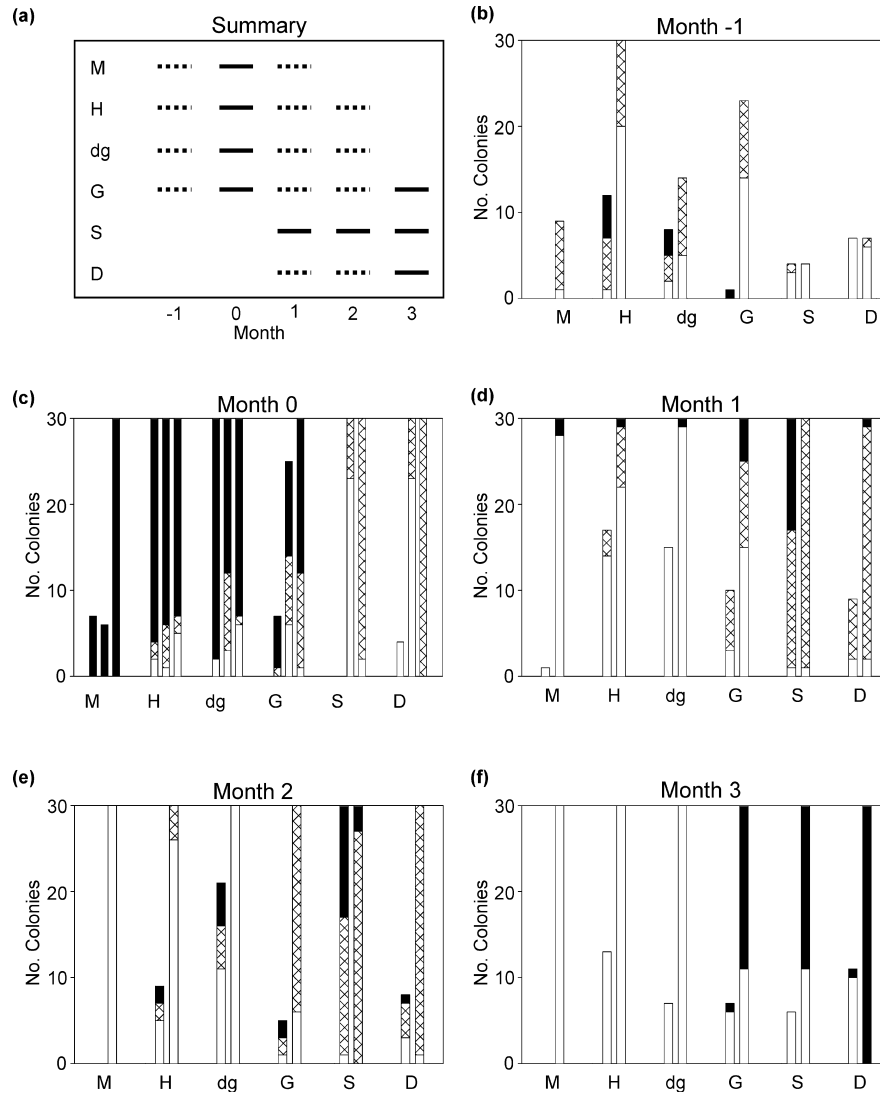
Fig. 3a–f Timing of egg maturity for the five species and the morph "dig-gem" of the *A.humilis* species group during the spawning season at Lizard Island. Months were standardised in relation to the month of mass spawning (month 0) as follows: *month* −1 November 1998, October 1999; *month 0* November 1999, November 2000, December 2001; *month 1* December 1999, December 2000; *month 2* January 2000, January 2001; *month 3* March 1999, February 2001. Horizontal axis is ordered by predominant spawning time, using the same codes for species and "dig-gem" as in Fig. 1. **a** Summary of timing of egg maturity for species and "dig-gem". *Absence of a line* indicates months when no colonies contained mature eggs; *dotted lines* indicate that <40% of colonies contained mature eggs; *solid lines* indicate that >40% of colonies contained mature eggs. **b–f** Stages of egg maturity for each month in the spawning season: *white* indicates that no eggs or extremely small eggs were present, *cross-hatching* indicates that immature eggs were present, *black* indicates the presence of mature eggs. *Multiple bars* for each taxon represent data recorded in different spawning seasons (years)

Spawning and fertilisation trials

Time (hour) of spawning and time taken for egg-sperm bundles to break apart were consistent for each species and morph, between different nights, months and years.

Most colonies of species and morphs spawning during the same month spawned over the same range of nights (Table 5). This suggests that the former two factors, hour of spawning and time taken for egg-sperm bundles to separate, play an important role in determining which morphs have the greatest potential to interbreed while "night of spawning" does not. Colonies of *A. humilis*, *A. gemmifera*, "hum-gem", "dig-gem" and "terete mont" spawned at similar times (2.5–3.5 h after sunset), while colonies of *A. monticulosa* and "mont-hum" spawned around 2–3 h later (Table 5). Following spawning, time taken for egg-sperm bundles to break apart varied substantially (Table 5), despite similar levels of agitation for spawn collected from each colony. Bundles released from colonies of "dig-gem" and some colonies of *A. gemmifera* broke apart almost instantaneously upon reaching the water surface. Bundles released from colonies of "hum-gem", "terete mont", *A. monticulosa*, "mont-hum" and some colonies of *A. gemmifera* broke up over a 0.5–2 h period. In contrast, time taken for bundles to separate from colonies of *A. humilis* ranged from 1–4 h. It therefore seems that all

Table 5 Observations of spawning in laboratory aquaria for species and morphs of the *Acropora humilis* species group recorded during three spawning seasons (1999–2000, 2000–2001 and 2001–2002)

Species or morph	Number of colonies spawning[a]	Number of colonies not spawning[b]	Month[c]	Night[d]	Time of spawning (hours after sunset)[e]	Separation of bundles[f]
A. samoensis	9	1	2, 3	3, 4	1.5–2	5–30 min
A. humilis	13	1	0	5, 6, 7, 8	2.5–3.5	1–4 h
A. gemmifera	9	0	0, 1, 3	6, 7, 8	2.5–3.5	5–30 min
A. monticulosa	9	1	0	6, 7, 8	5–6.25	30–60 min
A. digitifera	4	0	3	7	2.75	5–15 min
"Sam-hum"	2	3	2	4, 6, 7	2–2.5	30–60 min
"Sam-gem"	2	0	2	4, 6	1.5–2	< 5 min
"Hum-gem"	4	1	0, 2	6, 7, 9	2.5–3	30–90 min
"Mont-hum"	3	1	0	7, 8	5–6.25	30–60 min
"Terete mont"	2	0	0	7	2.5–3	1–2 h
"Dig-gem"	14	1	0	6, 7, 8	2.5–3.5	< 5 min

[a]Number of colonies spawning indicates the number of colonies spawning in aquaria, on which the observations in this table are based
[b]Number of colonies not spawning indicates the number of colonies in aquaria that did not spawn
[c]Month is month of spawning relative to the mass spawning (month 0); codes for the month of spawning are the same as those in Fig. 3
[d]Night is the number of nights after the full moon (night 0), on which spawning was recorded
[e]Spawning times ranged from 2030 hours to 0115 hours
[f]Separation of bundles indicates the time interval taken for egg-sperm bundles to be separated

species and morphs which spawned 2.5–3.5 h after sunset, except *A. humilis*, would be unlikely to breed with the later spawning colonies of *A. monticulosa* and "mont-hum". The longer time taken for bundles to separate for some colonies of *A. humilis* suggest a greater potential for this species to interbreed with *A. monticulosa* and "mont-hum", both of which released egg-sperm bundles that separated relatively quickly.

Although timing of spawning and time taken for egg-sperm bundles to break apart did not indicate opportunities for reproductive isolation between species and morphs which spawned after the mass-spawning event, timing of these traits do provide evidence of possible evolutionary connections for these taxa. Colonies of *A. samoensis*, "sam-hum", and "sam-gem" spawned 1.5–2.5 h after sunset and *A. digitifera* about 3 h after sunset (Table 5). Relative to colonies of *A. samoensis*, colonies of "sam-hum" spawned later and egg-sperm bundles took longer to break apart, while the bundles released from colonies of "sam-gem" separated more rapidly. These observations suggest a distinction between colonies of *A. samoensis* and the intermediate morphs "sam-hum" and "sam-gem", and a possible link with the species *A. humilis* and *A. gemmifera* respectively. Similarly, egg-sperm bundles released from colonies of *A. digitifera* separated rapidly, as seen for colonies of "dig-gem" (Table 5).

Preliminary observations in this study suggest that egg size may be related to timing of spawning, with smaller eggs being released from colonies in the second and third months after the mass spawning compared with eggs spawned during the mass-spawning month. This proposal is based on the finer plankton mesh needed to separate eggs and sperm from colonies that spawned in months 2 and 3. Measurement of spawned eggs from each species and morph, for each month that they spawn, is necessary to confirm these observations.

Levels of fertilisation between colonies of the same species were high to very high (usually >90%) and negligible between colonies of different species (Fig. 4). This suggests that strong pre-zygotic fertilisation barriers exist between species of the *A. humilis* species group that spawn on the same night. Levels of fertilisation between colonies of "dig-gem" were also high to very high and negligible between colonies of this morph and all other species and morphs, suggesting that fertilisation barriers are well developed between this morph and other synchronously spawning species and morphs. "Terete mont" was the only other morph that was tested for interbreeding potential because no other pairs of colonies of the same morph spawned on the same night. Although only two colonies were tested, the low to moderate levels of fertilisation between colonies of "terete mont" contrasts strongly with the high levels of fertilisation for all intraspecific and "dig-gem" crosses, suggesting that this morph may be a recent hybrid. Further crosses are necessary to explore this proposal. Extremely low levels of fertilisation were recorded between colonies of *A. humilis* and *A. monticulosa*, while moderate but never high levels of fertilisation were recorded between colonies of *A. humilis* and the morphs "mont-hum" and "terete mont", suggesting an evolutionary link between these species and morphs. Negligible levels of fertilisation between colonies of *A. monticulosa*, "mont-hum" and "terete mont" with colonies of *A. gemmifera* and "hum-gem" suggest strong reproductive barriers exist between these species and morphs. High levels of fertilisation between colonies of "hum-gem" and *A. gemmifera* and low levels with *A. humilis* and all other species and morphs with which colonies of "hum-gem" were crossed, suggest that this morph shares greatest affinity with *A. gemmifera*. Likewise, high levels of fertilisation between colonies of *A. samoensis* and "sam-hum" suggest a strong evolutionary affinity. Moderate fertilisation

levels between "sam-hum" and "sam-gem" reinforce the distinction between these morphs and *A. samoensis*, as is also evident in the timing of spawning and separation of bundles.
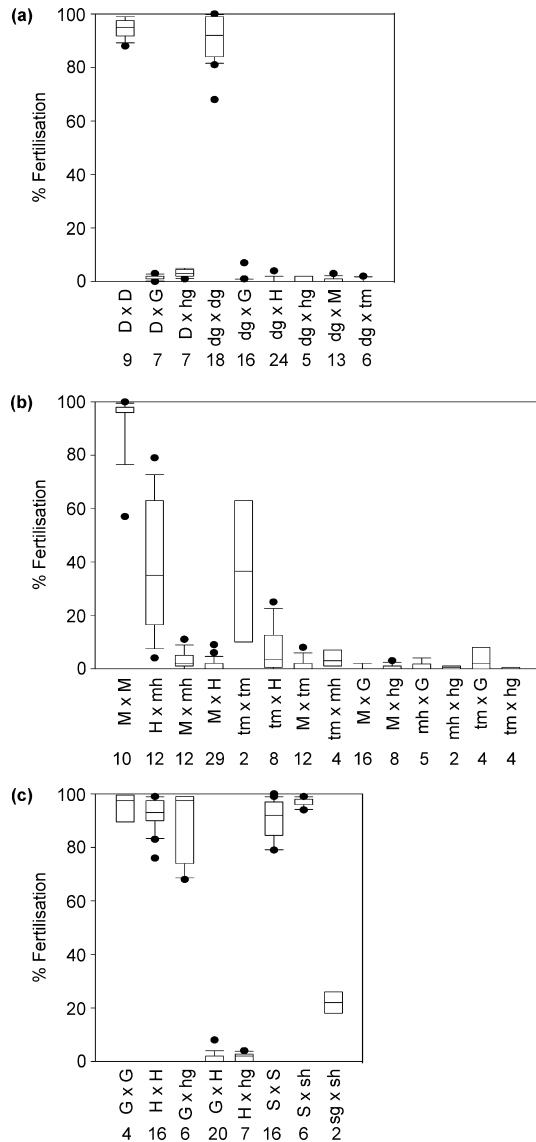
Fig. 4a–c Boxplots indicating the range of fertilisation levels (average of 3 replicates) for each cross that was tested. Interspecific and intermorph crosses are only presented in one figure. **a** "Dig-gem" and *A. digitifera*. **b** *A. monticulosa* and intermediate morphs of this species. **c** *A. gemmifera*, *A. humilis*, *A. samoensis* and intermediate morphs of these species. The *top* and *bottom of the boxes* represent the upper and lower quartiles (i.e. 50% of the data) with the *horizontal line* within the box indicating the median value; *whiskers* at either end of the boxes indicate values within 1.5 interquartile ranges and *dots* indicate extreme values beyond the *whiskers*. Codes for species and/or morphs for each pair of colonies used in each cross are indicated on the horizontal axis and are the same as those in Fig. 1. *Numbers below the codes* for each morph indicate the number of crosses tested.

Discussion

Evolutionary relationships within the *Acropora humilis* species group

The morphological, molecular and reproductive data presented in this study all contribute to the interpretation of evolutionary relationships between species and morphs in the *A. humilis* species group, with the five species and seven morphs at different stages of speciation. Predominant trends for each morph are summarised in Fig. 5. The molecular phylogenetic analysis (Fig. 2) provided the least resolution, indicating two levels of divergence for this marker. Divergence was largest between *A. digitifera* and "dig-gem" from other species and morphs of the *A. humilis* species group, while a more recent divergence of *A. monticulosa* was evident from the species *A. samoensis*, *A. humilis*, *A. gemmifera* and morphs of these species. The reproductive data provided greater resolution, indicating that temporal and prezygotic reproductive barriers are important mechanisms, maintaining and possibly structuring species boundaries in the *A. humilis* species group. Temporal reproductive isolation is apparent at the scale of months over the summer spawning season, based on timing of gamete maturity (Fig. 3) and hours within the mass spawning (Table 5). Evidence of reproductive isolation based on night of spawning was not apparent, with most colonies of species and morphs spawning during the same month, also spawning over the same range of nights (Table 5). High levels of intraspecific and negligible levels of interspecific fertilisation potential confirmed that the species of the *A. humilis* species group are valid species, while fertilisation potential between species and morphs corresponded with apparent evolutionary affinity based on morphological appearance.

Phylogenetic analysis of the mtDNA intergenic region resolved two distinct clades within the *A. humilis* species group, with sequences from colonies of *A. digitifera* and "dig-gem" forming clade I and sequences from all other species and morphs forming clade II (Fig. 2). This distinction between the two clades was corroborated by the results of the breeding experiments, which demonstrated that the potential for colonies of *A. digitifera* or "dig-gem" to interbreed with the species or morphs in clade II against which they were tested were negligible (Fig. 4a).

Within clade I, sequences from the colonies of *A. digitifera* and "dig-gem" were indistinguishable. Morphologically colonies of this species and morph are very similar, differing in that colonies of *A. digitifera* have thin branches compared with the thicker and more conical shaped branches of "dig-gem" (Table 1). Live colonies of this species and morph also share identical patterns of colour variation (Table 1). Considering the molecular and morphological similarities, it was surprising to discover that an almost complete temporal reproductive barrier separates this species and morph,

Molecular (Fig. 2)	Depth (Fig. 1)	Egg Maturity (Fig. 3)	Spawning Time (Table 5)	Bundle Separation (Table 5)	Fertilization Potential (Fig. 4)
A. digitifera	Shallow	After MS	Regular	Fast	-----------------
"dig-gem"	Shallow	MS	Regular	Fast	-----------------
"terete mont"	Shallow	MS	Regular	Moderate	Intra: moderate
A. monticulosa	Shallow	MS	Late	Moderate	-----------------
"mont-hum"	Shallow	MS	Late	Moderate	*A. humilis*: low
"mont-gem"	Shallow				
A. humilis	Shallow	MS	Regular	Slow - moderate	"mont-hum": low
"hum-gem"	Shallow	MS	Regular	Moderate	*A. gemmifera*: high
"sam-hum"	Deep	After MS	Regular	Moderate	*A. samoensis*: high / "sam-gem": moderate
A. gemmifera	Shallow	MS & After MS	Regular	Moderate	"hum-gem": high
"sam-gem"	Deep	After MS	Regular	Fast	"sam-hum": moderate
A. samoensis	Deep	After MS	Regular	Moderate	"sam-hum": high

Fig. 5 Summary of predominant patterns for each species and morph of the *A. humilis* species group, for molecular, ecological (habitat depth) and reproductive criteria. The figure or table from which each column is summarised is indicated under the heading of each column. The *tree at left* and *first column* of species and morph names represent a reduced version of the tree produced from the phylogenetic analysis of the mtDNA intergenic region. Each *row* in the remainder of the figure corresponds with the species and morphs listed within the tree. Molecular data were not obtained for "mont-hum" or "mont-gem": these morphs are placed in *dashed boxes* to separate them from the molecular tree. Depth is coded as *shallow* or *deep*, indicating the relative depth of the habitat in which species and morphs most commonly occurred. Egg maturity is coded as follows: *After MS* after mass spawning, *MS* mass spawning. Spawning time is coded as *regular* or *late*, relative to the hour of spawning of other species and morphs; bundle separation is coded as *fast, moderate* or *slow*, relative to time taken for bundles to break apart for colonies of each species and morph; fertilisation potential indicates which species and morphs showed potential to interbreed, based on the fertilisation data, and are coded as *low, moderate* or *high*; the morph "terete mont" showed moderate intra-morph levels of fertilisation while all other species or morphs had high levels of intraspecific or intra-morph levels of fertilisation; *A. digitifera*, "dig-gem" and *A. monticulosa* showed no or extremely low potential to interbreed with other species or morphs; no reproductive observations were recorded for "mont-gem"

with "dig-gem" predominantly spawning during the mass-spawning month and *A. digitifera* spawning 3 months after the mass spawning. These differences in timing of spawning indicate that *A. digitifera* and "dig-gem" are unlikely to interbreed at Lizard Island, although fertilisation experiments are necessary to test whether they could interbreed if colonies spawned at the same time. The other species with which the morph "dig-gem" shares morphological characters is *A. gemmifera*, which also has conical shaped branches and radial corallites whose shape merges with that of "dig-gem" (Table 1). No temporal reproductive barrier was evident between "dig-gem" and *A. gemmifera*, with both spawning simultaneously during the mass spawning. However, inter-fertilisation potential was negligible (Fig. 4a), indicating that a pre-zygotic barrier reproductively isolates them. Based on the combined evidence of the morphological, molecular and reproductive results, three hypotheses could explain the origin of "dig-gem". Firstly, this morph may have evolved from within the species *A. digitifera* due to or reinforced by a differential timing of spawning. A second hypothesis is that *A. digitifera* evolved from "dig-gem", also as a result of or reinforced by a differential timing of spawning. A third hypothesis is that "dig-gem" may be derived from *A. digitifera* and *A. gemmifera*, through hybridisation. Hybridisation between *A. digitifera* and *A. gemmifera* would be most likely to occur in the third month after the mass spawning when most colonies of *A. digitifera* and many colonies of *A. gemmifera* spawned. Irrespective of the evolutionary origin of "dig-gem", it now comprises a discrete evolutionary unit, which is more abundant than any other species or morph of the *A. humilis* species group at Lizard Island (Fig. 1). Analysis of molecules involved in gamete recognition, e.g. bindin or lysin (Palumbi 1994), would be most useful for testing each of the three hypotheses and resolving the evolutionary origin of "dig-gem".

Within clade II of the molecular phylogenetic analysis, sequences from colonies of *A. monticulosa* grouped in a subclade (clade IIa) with high bootstrap support (95%), suggesting that this species has also diverged from other species within the *A. humilis* species group (Fig. 2). This apparent divergence of *A. monticulosa* is congruent with the extremely low potential of this species to interbreed with other species in clade II (Fig. 4), as well as the late spawning time of this species (Table 5) forming a temporal reproductive barrier from other mass-spawning species. Colonies of *A. monticulosa* were the most synchronised in timing of spawning compared with all other species within the *A. humilis* species group, spawning almost exclusively in the mass-spawning month during a time interval of just over 1 h. This species consistently spawned 5–6.25 h after sunset, while most species of *Acropora* spawn 2–3.5 h after sunset, as recorded in this study (Table 5) and by Babcock et al. (1986), with the latest previous recorded spawning time for any species of *Acropora* being 3.8 h after sunset (Babcock et al. 1986). The separation in timing of spawning of *A. monticulosa* from other species monitored in this study by 2–3 h, and 1–2 h after the latest time recorded for any other congeneric species provides convincing evidence that this species is reproductively isolated by temporal barriers. In comparison, temporal reproductive isolation has also been proposed for other species of *Acropora* (van Oppen et al. 2001; Fukami et al. 2003) with separation times ranging from 0.5–3 h (Babcock et al. 1986; Hayashibara et al. 1993; Fukami et al. 2003). Temporal reproductive isolation has also been suggested in the *Montastraea annularis* species complex, with *M. franksi* spawning 1–2 h earlier than *M. annularis* and *M. faveolata* (Knowlton et al. 1997; Szmant et al. 1997).

The species *A. monticulosa* appears to share greatest evolutionary affinity with *A. humilis*, with several factors supporting this proposal. It is feasible that the prolonged period of separation for egg-sperm bundles released from colonies of *A. humilis*, and the later spawning time of *A. monticulosa* (Table 5) has provided or maintained an opportunity for these species to continue to interbreed, that does not exist for *A. monticulosa* and other species of the *A. humilis* species group. Colonies of the morph "mont-hum" were the only other colonies to spawn at the later time recorded for *A. monticulosa*, while "mont-hum" egg-sperm bundles also separated over a 30–60 min interval (Table 5). Levels of fertilisation between *A. monticulosa* and colonies of *A. humilis* and morphs of these species were extremely low, but slightly higher than levels with any other species or morphs, supporting the proposed divergence of *A. monticulosa* but closest evolutionary affinity with *A. humilis*. Meanwhile, moderate levels of fertilisation were recorded between colonies of *A. humilis* and the morphs "mont-hum" and "terete mont". Based on these fertilisation records and the morphological affinities of these morphs, it is possible that they may be of hybrid origin, derived from *A. humilis* and *A. monticulosa* and able to

backcross with colonies of *A. humilis* but rarely with colonies of *A. monticulosa*. The low to moderate levels of fertilisation between two colonies of "terete mont", in contrast to the high levels of fertilisation for all other intra-species and intra-morph crosses provides additional evidence that this morph may be a hybrid, in which sterility barriers partially reduce the potential for colonies of this morph to inter-breed. Further support for the hybrid origin of the morph "terete mont" is indicated in the molecular phylogeny, with the sequence for this morph having low bootstrap support and grouping within neither clade IIa nor IIb.

Sequences from colonies of *A. humilis*, *A. gemmifera*, *A. samoensis* and morphs of these species formed a second subclade (IIb) also with high bootstrap support (95%), in the molecular phylogenetic analysis (Fig. 2). There was little further differentiation between sequences within this subclade, suggesting a close evolutionary relationship between these species relative to *A. digitifera* and *A. monticulosa*. The existence of intermediate morphs between *A. humilis*, *A. gemmifera* and *A. samoensis* also suggests a close relationship between these species, with the morphological continuum between *A. humilis* and *A. gemmifera* [this study and Wallace (1999)] and molecular evidence (Wolstenholme et al. 2003) suggesting these two species are closely related. The consistent late maturation of gametes in colonies of *A. samoensis* also suggests this is the most distinct of the three species. Greatest evolutionary affinity of "sam-hum" and "sam-gem" with *A. samoensis* is suggested by morphological similarity and supported by the same late maturation of gametes in the second and third months after the mass spawning. High levels of fertilisation between *A. samoensis* and "sam-hum" suggest reproductive barriers have not formed between this species and morph. Meanwhile, substantially lower levels of fertilisation between "sam-hum" and "sam-gem" suggests that their morphological differences reflect the reduced breeding compatibility and a possible hybrid status for these morphs.

Further investigation of egg size may contribute to understanding factors determining timing of spawning. Preliminary evidence from this study indicates that the relatively small eggs in the species *A. samoensis* and *A. digitifera* may be related to timing of spawning. These species do not appear to be closely related, based on the molecular data and morphological appearance. They also differ in the habitats that they occupy, with *A. samoensis* mostly occurring in slope and lagoonal habitats and *A. digitifera* on reef flats. Therefore, the smaller eggs in these species do not appear to be attributable to environmental conditions. Measurement of eggs released from colonies of each species and morph, particularly those that spawned in different months, would clarify whether there is a correlation between egg size and species/morph or timing of spawning. Such patterns may have been overlooked in previous studies, which only measured egg size in preserved branch samples, irrespective of timing of egg maturity and spawning.

Taxonomic implications

Species of corals are well known to be morphologically variable, with boundaries between many species remaining unclear (e.g. Lang 1984). This study demonstrates the value of working at the morph level for clarifying evolutionary boundaries in corals. Recognition of morphs within or between species reduces taxonomic error as a result of "forcing" colonies into incorrect or inappropriate species categories. The most outstanding case in this study is the morph "dig-gem", which was substantially more abundant than any other species or morph at Lizard Island. According to the current taxonomy, this morph could have been identified as either *A. digitifera* or *A. gemmifera* (Table 1), but it is distinct from both of these species. Identification of this morph as either of these species would conceal important evolutionary distinctions between these species. If colonies of "dig-gem" were identified as *A. digitifera*, it would appear that this species spawns from month −1 to month 3, with no temporal reproductive barrier isolating it from other species and morphs of the *A. humilis* species group. Conversely, if colonies of "dig-gem" were identified as *A. gemmifera*, DNA sequences for the marker examined in this study would be present in both clades I and II for this species, and fertilisation levels would range from 0% to 100%. In the case of the other species examined in this study, the most serious consequences of not distinguishing the species and morphs would be the interpretation of substantially broader levels of fertilisation for each species, which in many cases would range from 0% to 100%.

Accurate identification of morphological species and morphs provided the foundation for interpreting relationships between the species and morphs examined in this study. This is in contrast to the conclusions of van Oppen et al. (2001), who state that "skeletal morphology may have been effectively uncoupled from the genotype in the case of *Acropora* evolution" and in the case of *A. humilis*, "morphology may have arisen several times independently". The current study demonstrates that close examination of morphological boundaries, using molecular and breeding criteria, is a powerful technique for resolving evolutionary boundaries in corals, as proposed by Willis (1990), Stobart (2000) and Wolstenholme et al. (2003). In addition, the morphs provided a valuable tool for testing possible evolutionary links between species, while the absence of intermediate morphs between other species, e.g. between *A. monticulosa*, *A. samoensis* and *A. digitifera*, corresponds with the low potential of these species to interbreed. This is the first study to assess the potential of species of the *A. humilis* group to interbreed, and therefore comparison with other studies that have tested fertilisation potential under laboratory conditions is not possible for these species. Sperm competition experiments would provide a further test of prezygotic barriers and therefore evolutionary boundaries (Márquez et al. 2002a), between species and morphs. This could be done for pairs of species and morphs, which showed no potential to interbreed, to confirm the existence of prezygotic barriers. In cases where there was potential for interbreeding, for example between *A. gemmifera* and "hum-gem", sperm competition experiments would establish whether fertilisation potential varied between the same and different morphological groups.

Intermediate morphologies and breeding potential are not conclusive evidence of hybrid status. For example, many hybrids exhibit extreme (positive or negative) phenotypic characters relative to parent species (reviewed in Rieseberg et al. 1999). To confirm the evolutionary affinities of morphs recognised in this study, it will be necessary to examine the species and morphs using a combination of nuclear and mitochondrial molecular markers. This has recently been demonstrated for *A. prolifera*. At least two morphs of *A. prolifera*, both of which are morphologically intermediate between the species *A. cervicornis* and *A. palmata*, are now known to be of hybrid origin and derived from these species (Vollmer and Palumbi 2002). Examination of chromosome number is also likely to contribute to understanding evolutionary relationships between the species and morphs examined in this study. Kenyon (1997) concluded that polyploidy, resulting from the combination of sets of chromosomes from different species during hybridisation events is a likely source of gametic incompatibility between species in the genus *Acropora*. Evaluation of chromosome numbers in species as well as morphs may therefore also provide an important tool for tracing evolutionary relationships within the genus *Acropora* and possibly other groups of corals.

The variation between sequences within individual colonies was surprising, given that sequences from mitochondrial markers are not expected to vary at this level (Avise 2000). Two patterns contributed to this variation. Firstly, although repetitive sequences are a typical feature of control regions (van Oppen et al. 2002a and references within), the occurrence of repeat-sequence blocks in this study was not consistent within individuals. Repeat-sequence blocks have been reported for other species of *Acropora*, but no other cnidarians for which data are available (van Oppen et al. 2002a), while patterns of intra-individual variation have not been examined in other studies of cnidarians. Further research is necessary to understand the evolutionary significance of these repeat regions (van Oppen et al. 2002a) and whether they have the potential to contribute to the interpretation of evolutionary relationships in the genus *Acropora* (Wolstenholme et al., article in preparation). Secondly, differences in sequences (excluding the repeat regions) from single individuals, varied by up to 10 bp (Table 2). Although some of this variation is likely to be due to PCR error (Saiki et al. 1985), sites which varied as a result of PCR error and those that are due to intra-individual polymorphisms cannot be distinguished. Further interpretation of this variation, within an evolutionary context, is therefore not possible.

This study was restricted to the *A. humilis* species group at Lizard Island on the Great Barrier Reef, Australia. Species within this group and other species groups in the genus *Acropora* were arbitrarily assigned based on apparent morphological similarity (Veron and Wallace 1984; Wallace 1999). It is possible that the species and morphs in this species group also share evolutionary affinities with other species in the genus *Acropora*. This particularly applies to *A. digitifera*, given the clear distinction between this species and other species of the *A. humilis* species group in this study. Phylogenetic analysis of morphological characters of the genus *Acropora* suggests that *A. digitifera* may be most closely related to species of the *A. nasuta* and *A. divaricata* species groups (Wallace 1999). Broader analyses, which examine additional species within the genus *Acropora* using complementary techniques as in this study, are necessary to explore this possibility. It is likely that the status of evolutionary relationships between species and morphs will vary in different geographic locations (e.g. Hayashibara and Shimoike 2002). Therefore, such projects must also be conducted at a broad geographic scale before the taxonomic status of the morphs and the boundaries of current species can be fully resolved.

This study demonstrates that morphology is a valuable tool for interpreting evolutionary relationships in the *A. humilis* species group. This is likely to also be true for other species of *Acropora* and other coral taxa. In particular, morphs may indicate active zones of speciation (e.g. between *A. humilis* and *A. gemmifera*) or hybridisation (e.g. between *A. digitifera* and *A. gemmifera*), which can then be tested using genetic and reproductive criteria. Confirmation of morphology as an informative character of evolutionary boundaries is of great significance because most coral research projects rely on morphology as the primary tool for recognising species.

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