Marine Biology (1997) 127: 705–711 © Springer-Verlag 1997

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Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panamá and Honduras)

Received: 22 July 1996 / Accepted: 2 October 1996

Abstract *Montastraea annularis*, *M. faveolata*, and *M. franksi* are three recently separated species that together dominate reefs of the tropical western Atlantic. Despite morphological, life-history, ecological and genetic differences, the legitimacy of their status as separate species has been questioned. This controversy stems from both the scarcity of unambiguous, diagnostic differences among them, and from the possibility of extensive hybridization associated with their approximately synchronous reproduction in sympatry. Here we report on fertilization trials and the timing of spawning, both of which suggest that the potential for hybridization may be limited. Crosses between *M. faveolata* (the most genetically distinctive taxon) and the other two species were largely unsuccessful, as were selfed matings for all three species. *M. annularis* and *M. franksi* showed no evidence of fertilization barriers, but the timing of spawning typically differed between them by 1 to 2 h. We also found that spawning times in the field of *M. annularis* and *M. faveolata* were non-overlapping in 1995, and that the timing of spawning in *M. annularis* could be experimentally shifted forward by simulating earlier than natural sunsets. These findings from Panamá and Honduras, particularly given their consistency with comparable observations and experiments elsewhere, provide evidence of reproductive isolation and support the separate species status of these three taxa. Some hybridization may occur under natural conditions at these sites, particularly between *M. annularis* and *M. franksi*. Rampant genetic interchange among these taxa in Central America seems unlikely, however, in the light of the concordant associations between morphology, reproductive biology and other characters which are observed.

Communicated by N.H. Marcus, Tallahassee

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Introduction

Montastraea annularis sensu lato is the most important and best-studied reef builder in the tropical western Atlantic. For many years, extensive variation in colony morphology was believed to be due to non-genetic phenotypic plasticity. However, recent studies have revealed a variety of differences in corallite structure, distribution, life history and allozymes that are concordant with differences in colony morphology (Knowlton et al. 1992; Van Veghel and Bak 1993). On this basis, Weil and Knowlton (1994) argued that there were at least three species commonly found in shallow to intermediate depths, and resurrected two previously synonomized forms (*M. faveolata* and *M. franksi*).

These newly resurrected species have appeared in a recent guide to the corals of the region (Humann 1993), but acceptance of their separate status has not been universal. Van Veghel and Bak (1993) argued against recognizing them as separate species in the absence of fixed, diagnostic differences. Veron (1995) suggested that this controversy was typical of corals in general and stated that "... there may be no 'correct' answer because species are arbitrary..." in corals. Species are not arbitrary, however, if reproductive isolation can be documented. Reproductive isolation is the defining character for biological species and a suitable diagnostic character for phylogenetic species (Knowlton and Weigt 1997).

Like many major reef builders, members of the *Montastraea annularis* complex participate in annual mass spawnings concentrated on just a few nights each

year. Both direct observations and histological studies indicate that most spawning takes place in the second half of August or the first half of September ≃ 7 to 8 d after the full moon [albeit with some latitudinal and year-to-year variation; see Van Veghel (1994) and references therein]. Thus, reproductive isolation, if it exists, must involve either direct barriers to fertilization or development, or relatively subtle differences in the timing of spawning. Van Veghel (1994) observed spawning of the three taxa in Curaçao and failed to find any evidence for temporal barriers to hybridization, but he was unable to perform cross-fertilization experiments.

Resolving this taxonomic controversy is particularly important, given the ecological dominance of the *Montastraea annularis* complex throughout the Caribbean and its role as a model system for many studies of coral biology. To assess the extent of reproductive isolation among the three species, we recorded the dates and times of spawning and performed artificial crosses during the annual spawning periods of 1994 and 1995.

Materials and methods

Studies of spawning in the *Montastraea annularis* species complex were based on laboratory observations and experiments in Panamá (August 1994, August and September 1995) and on field observations in Honduras (August 1995).

Panamá

Spawning behavior was observed in corals from the San Blas Archipelago while collecting gametes as a source of DNA for genetic studies. Large colonies of the three species that appeared to be ripe were collected from two adjacent reefs at the eastern end of the Cayos Limones [9°33′N, 78°53′W; just east of Site LM in Fig. 1 of Weil and Knowlton (1994)], generally from depths of 3 to 12 m. Collections came from corals separated by several meters, and previous electrophoretic studies had indicated that inadvertant collection of clonemates was unlikely (Knowlton et al. 1992). Ripeness was judged by removing a small chip with a hammer and chisel from the central part of the colony and looking for pinkish eggs, which are visible to the naked eye and conspicuous when viewed with a hand-lens underwater. After collection, colonies were brought to the Smithsonian San Blas station, cleaned of obvious epifauna and epiflora on dead surfaces, and kept under docks (to avoid excess light) in depths of about of ≃ 1 m. On the evenings of observation just prior to sunset, colonies were moved to 20-liter buckets or larger tanks (no more than one colony per container) containing seawater filtered with a 100 μm plankton net. Corals were kept away from artificial lights and were checked with small headlights for the appearance and release of egg/sperm bundles at ≃ 20 to 30 min intervals. Containers were partially flushed at about 20:30 hrs, and corals that did not spawn heavily were returned to the sea between 23:00 hrs and midnight. Only approximate times of spawning were recorded in 1994, but differences between *Montastraea franksi* and the other two species were nonetheless notable. In 1995, precise times for the initiation of spawning were recorded. The dates and times of collections, monitoring, full moons and sunsets are summarized in Table 1.

In September 1995, we simulated early sunsets to test Van Veghel's (1994) suggestion that falling light levels are the proximate cue controlling the timing of spawning in these corals. We took advantage of the columnar branching morphology of *Montastraea annularis* and collected two columns from each of eight colonies. One member of the pair was designated as the experimental column and the other its clonemate control. Experimental columns were darkened by covering them with two black plastic garbage bags between 16:30 and 17:00 hrs (roughly 2 h prior to sunset), while control columns were left uncovered but otherwise treated identically. Columns used in this experiment were maintained in one of two ways.

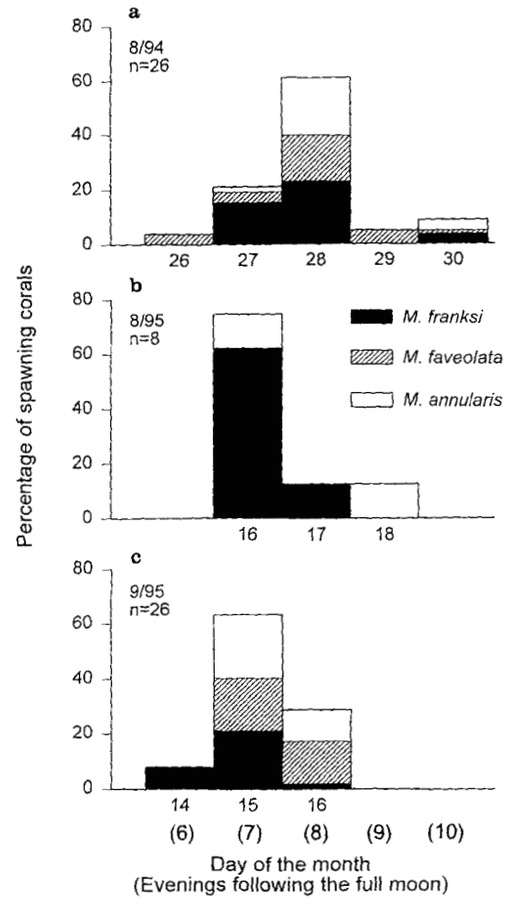

Fig. 1 *Montastraea* spp. Species composition for peak spawning periods of August 1994 (**a**), August 1995 (**b**) and September 1995 (**c**) in Panamá (*n* total number of spawning corals for all species combined; percentages for each graph sum to 100). Actual dates for each month (*abscissas*) are aligned (with number of evenings following full moon shown in parentheses below abscissa of Graph **c**). Corals were also checked for spawning prior to first dates recorded in this figure (see Table 1)

Table 1 Astronomical data and collecting and monitoring dates for Panamanian spawning studies of *Montastraea* spp. Collections were largely completed prior to onset of spawning, but dates of collection and monitoring overlapped because of conflicting aims of having large numbers of corals and some freshly collected corals in the laboratory whenever spawning occurred

Full moon		Sunset time (hrs)	Collection dates	Monitoring dates
date	time (hrs)			
21 Aug. 1994	01:47	18:31	19–29 Aug. 1994	20–31 Aug. 1994
10 Aug. 1995	13:15	18:36	12–17 Aug. 1995	13–18 Aug. 1995
8 Sep. 1995	22:37	18:21	12–14 Sep. 1995	13–17 Sep. 1995

Columns from four of the colonies were kept in 1 m of water with the other collected colonies and were treated similarly, apart from earlier removal into buckets and times of flushing (19:00 and 21:00 hrs). Both bagged and unbagged buckets were kept in the shade from 16:30 to 18:30 hrs to avoid overheating. The other four pairs were kept in 29 × 29 × 44 cm aquaria for the duration of the experiment. Between 16:30 and 23:30 hrs, water was flushed twice (as above), and at other times was slowly circulating. The first day of bagging was 13 September 1995 for columns maintained in aquaria, and 14 September 1995 for columns transferred to buckets each afternoon.

We also performed a series of crossing experiments in September 1995. On the night of 15 September two colonies of each of the three species were selected for a balanced mating design of all possible combinations, including selfing. One egg/sperm bundle from each colony (or two from the same colony for selfing experiments) was collected using an unused 10 ml serological pipette, and placed in a 120 ml urine specimen cup (6 cm diam at top) that had been washed and soaked in seawater. Water was changed and eggs or larvae checked daily for several days. A similar experiment was started on 16 September; conditions were identical except that 20 egg/sperm bundles (rather than one) from each source colony were placed in the cups. These fertilization trials with higher gamete densities involved four colonies of *Montastraea annularis* four of *M. faveolata*, and one of *M. franksi*, but no attempt was made to make all possible pairwise crosses.

Honduras

On 13 August 1995, 10 colonies of each of the three species were tagged on a fringing reef dominated by *Montastraea annularis* sensu lato (at least 80% of coral cover). Depths ranged from 3 to 4 m for *M. annularis* sensu stricto and *M. faveolata*, and from 4 to 11 m for *M. franksi*. Rough weather precluded evening observations from 13 to 15 September, but divers monitored marked corals on 16 September from 17:30 to 22:30 hrs.

Results

Spawning dates, Panamá

In 1994, we had relatively little difficulty in finding ripe specimens of *Montastraea* spp. shortly before the late-August full moon, and most of the corals we collected subsequently spawned. Maximum spawning was observed on 28 August (Fig. 1a), eight evenings after the full moon, with the second heaviest spawning occurring 1 d earlier.

In 1995, the full moon in August was 11 d earlier than in 1994, and we had much greater difficulty finding apparently ripe corals in the field at this time. Of the 18 we collected, only 8 spawned: 6 of 7 *Montastraea franksi*, 2 of 7 *M. annularis* and 0 of 4 *M. faveolata*. Maximum spawning occurred on 16 August, (Fig. 1b), seven evenings after the full moon. In contrast, the following month many colonies appeared ripe, and nearly all collected corals spawned (including those that were collected in August but had not spawned in that month). Maximum spawning occurred on 15 September (Fig. 1c), seven evenings after full moon. There was also substantial spawning on 16 September.

Spawning dates, Honduras

In 1995, all previously marked colonies (and many others) of *Montastraea annularis* and *M. faveolata* spawned on 16 August (the only night that weather permitted observations to be made). In contrast, no marked colonies and only one unmarked colony of *M. franksi* was observed spawning that night.

Spawning times

The pattern of gamete release by individual colonies that we observed resembled that recorded by Van Veghel (1994). Shortly before spawning, the egg/sperm bundles could be seen at the openings of the polyps ("setting"). Gametes were released relatively quickly, with the bulk of spawning by a colony taking place within a 15 to 30 min interval. The majority of colonies appeared to release most of their gametes on a single night.

In Panamá in 1995 (Fig. 2a, b), *Montastraea franksi* typically spawned in buckets between 20:00 and 21:00 hrs, or 1.5 to 2.5 h after sunset. In contrast, most spawning of *M. faveolata* and *M. annularis* occurred between 22:00 and 23:00 hrs, or 3.5 to 4.5 h after sunset, with a number of the *M. faveolata* spawning before the first spawning by *M. annularis*. These differences in the distributions of spawning times are statistically significant ($p < 0.001$, Kruskal–Wallis one-way ANOVA), and also match our less precise notes from 1994 (20:15 to 21:00 hrs for *M. franksi*; 21:30 to 22:30 hrs for *M. faveolata* and *M. annularis*).

In the field in Honduras (16 August 1995), the only *Montastraea franksi* observed to spawn did so at 18:15 hrs. Massive spawning by *M. faveolata* occurred from 19:45 to 20:00 hrs, followed by a similarly major release of gametes by *M. annularis* from 21:30 to 22:10 hrs. The site in Honduras is in a time zone 1 h earlier than Panamá, but differs in longitude by only $\simeq 7.5°$ (yielding a sunset at $\simeq$ 18:00 hrs local time), so that these observations roughly correspond to 0.25 hrs, 1.75 to 2.0 hrs and 3.5 to 4.0 hrs after sunset, respectively. Thus *M. franksi* and *M. faveolata*, but not *M. annularis*, spawned >1 h earlier relative to sunset in Honduras (field) than in Panamá (laboratory).

Subsequent to this study, observations of spawning times of the three species were made in the field in Panamá on 4 September 1996. They did not differ from times observed in the laboratory for 1994, 1995, or 1996.

Artificial sunset

Montastraea annularis columns that were darkened early consistently spawned before the control columns (Fig. 2c) ($p < 0.001$, Mann–Whitney U-test). Spawning times of the controls resembled those observed for *M. annularis* colonies that were not put in buckets until 18:00 to 18:30 hrs (Fig. 2b,c). All eight artificially darkened columns spawned on the night of maximum spawning (15 September 1995), and some colonies spawned again on the following night. Only 2 of 8

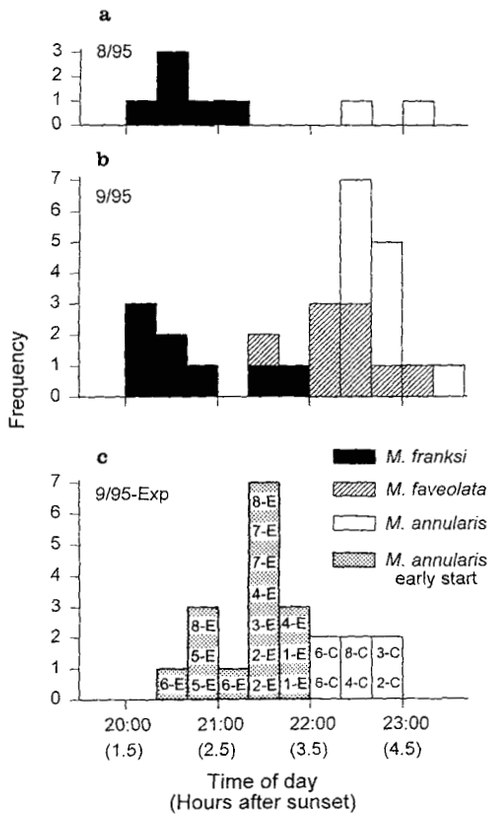

Fig. 2 *Montastraea* spp. Timing of spawning of corals collected in Panamá. **a, b** Corals experiencing natural sunsets; for corals that spawned on more than one day, time associated with maximum spawning is indicated. **c** Effects of accelerating sunset (*early start*) on experimental (*E*) columns from eight colonies, compared to control (*C*) columns from same colony. Both times are indicated for columns that spawned on more than one day, although statistical analyses (see "Results – Spawning times") are based only on the primary spawning time to avoid lack of independence. Colonies 1 to 4 were maintained in aquaria, while Colonies 5 to 8 were stored during the day in the field (see "Materials and methods – Panamá"). Hours after sunset are given in parentheses under abscissa of Graph **c**

control columns spawned on 15 September; one of these spawned again on 16 September, in addition to three which spawned for the first time. One control column spawned so little that gametes were not seen until the next morning, and two never spawned in detectable amounts. For the five colonies for which both experimental and control columns spawned, the median difference in spawning time was 65 min.

Crossing experiments

In the experiment involving all pairwise and selfing crosses for two colonies of each of the three species, it was difficult to distinguish fertilized from unfertilized eggs on the morning after spawning. By the second morning, however, successful fertilizations followed by early development could be readily recognized (swimming embryos/larvae, either shaped like planulae or round with a blastopore). Counts of these larvae yielded clear and consistent patterns (Fig. 3). Selfing crosses of *Montastraea faveolata* and *M. franksi* had no living larvae, and the maximum observed for selfed *M. annularis* was four. In contrast, non-selfed, intraspecific crosses yielded 41 to 110 larvae. Interspecific crosses between *M. annularis* and *M. franksi* were also successful at this stage, with numbers ranging from 55 to 129 larvae. Interspecific crosses involving *M. faveolata*, on the other hand, had no living larvae in 4 of 8 crosses, with a maximum of 15 larvae.

Crosses made with 20 egg/sperm bundles per colony were consistent with these results. One selfing experiment with *Montastraea faveolata* yielded no living larvae by the second day. Intraspecific crosses involving *M. annularis* or *M. faveolata* (4 colonies combined three ways in each case) yielded between 100 and 200 larvae. Three interspecific crosses between *M. annularis* and *M. franksi* (using 3 and 1 colonies, respectively) yielded $\simeq 100$ larvae each. In contrast, two crosses between *M. annularis* and *M. faveolata* (using 1 and 2 colonies, respectively) yielded only 6 and 21 larvae.

Taken together, these experiments exhibited two non-overlapping classes of outcomes. The 7 selfed matings using all three species and the 10 interspecific crosses involving *Montastraea faveolata* always produced fewer than 25 living larvae after 2 d, with half or more producing no living larvae at all. In contrast, the 9 intraspecific crosses involving all three species and the 7 crosses between *M. annularis* and *M. franksi* always produced ≥ 40 larvae. We cannot calculate the percentage of eggs fertilized in these experiments, because un-

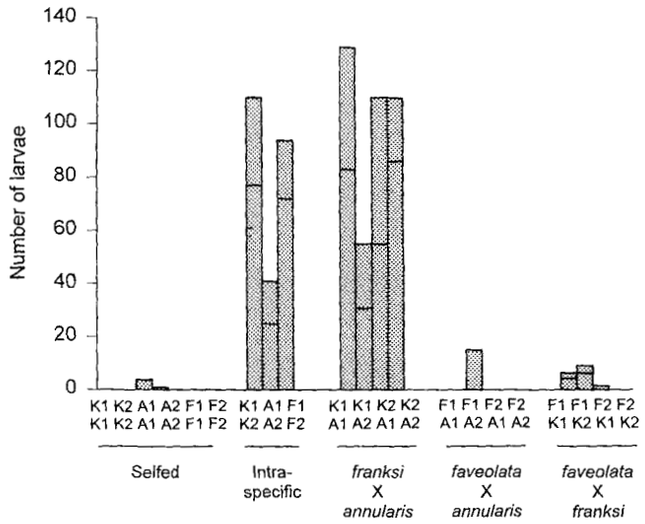

Fig. 3 *Montastraea* spp. Number of living larvae observed 2 d following artificial crosses involving two colonies of each of the three species (*K*: *M. franksi*; *A*: *M. annularis*; *F*: *M. faveolata*). Each of the 21 crosses was performed twice (each replicate with one egg/sperm bundle from each colony); where both yielded living larvae, totals are separated by line within bar. Comparable results for 12 additional crosses using 20 bundles per colony are described in "Results – Crossing experiments"

fertilized eggs decomposed and gamete bundles vary in the number of eggs they contain (Szmant et al. 1997). However, the bundles were distributed randomly with respect to treatment, so that these patterns are unlikely to be associated with initial differences among bundles in egg numbers.

Discussion

Caveats

Research on mass spawning is constrained by the very limited amount of time per year available for study. The sample sizes reported here are consequently small, but many of the patterns observed (low selfing, earlier spawning by *Montastraea franksi*, interfertility of *M. annularis* and *M. franksi*, barriers between *M. faveolata* and the other two species) have been noted in other regions (Szmant et al. 1997; D. Hagman et al. in preparation). We cannot directly estimate how representative our collections were of all corals in the region, because we only collected and observed those that appeared ripe. However, data from elsewhere (Van Veghel 1994; Szmant et al. 1997; D. Hagman et al. in preparation) and previous histological studies from Panamá (Soong 1991) strongly suggest that most spawning by these three species in 1994 and 1995 took place during our monitoring periods. Moreover, field studies of fertilization of the mass-spawning *Montipora digitata* on the Great Barrier Reef suggest that fertilization is drastically reduced on all nights other than the night of peak spawning (Oliver and Babcock 1992). Thus, most successful reproduction in 1994 and 1995 very probably occurred during the periods we monitored.

Spawning dates

In Panamá, the three species exhibited the same modal dates of spawning (Fig. 1), but *Montastraea franksi* showed a tendency towards spawning more heavily both in the earlier of the two possible months in 1995 (75% of all August spawners) and on the night preceding peak spawning (27 August 1994 and 14 September 1995), compared to the other species. D. Hagman et al. (in preparation) report similar trends. In Honduras, peak spawning by *M. annularis* and *M. faveolata* clearly occurred on the one night that weather allowed observations to be made. We do not know when or if the bulk of the *M. franksi* spawned in 1995, but if there was a major spawning, it was apparently not at the same time as *M. annularis* and *M. faveolata*

Spawning times

The distinct temporal isolation in spawning between *Montastraea franksi* and the other two species reported here for Panamá has also been documented for the Florida Keys (Szmant et al. 1997) and the Texas Flower Gardens (D. Hagman et al. in preparation). Observations from these three sites also indicate that *M. faveolata* tends to spawn earlier than *M. annularis*, although some individuals of the latter often begin spawning before spawning by *M. faveolata* is complete. The single observation in Honduras documented a similar but more marked temporal difference between these two species. Thus, whenever temporal differences have been observed, the order of spawning for the three species has been the same. Curaçao (Van Veghel 1994) is the only site where species were distinguished in the field, but no differences in spawning times were recorded.

Intraspecific synchrony and temporal differentiation between species might be increased by pheromonal stimuli. If pheromonal communication plays an important role, one would expect a broader range in spawning times among widely separated colonies in the field or isolated colonies in the laboratory.

Our resetting of the spawning clock by simulating an early sunset for several days before spawning provides experimental support for Van Veghel's (1994) suggestion that sunset is the short-term cue responsible for the timing of gamete release on the night of spawning (although controls for possible effects of bagging per se should be included in future studies). The apparent response of *Montastraea annularis* to premature darkening is similar to that observed for several other corals (Babcock 1984; Harrison 1989; Hunter 1989). Use of artificially long or short day lengths may have experimental utility in future fertilization studies.

The effect of apparent sunset on gamete release may also partially explain the earlier spawning seen in Honduras in the field compared to Panamá, where corals were kept in artificially shallow water and placed in buckets before sunset. Presumably corals are responding to low light levels rather than sunset per se, so that corals at greater depths should perceive "sunset" as occurring earlier (an hypothesis that could be readily tested). The effects of collection and handling per se may also have contributed to the differences observed between Panamá and Honduras. The reason why *Montastraea annularis* did not show the same pattern of earlier spawning in Honduras remains unclear.

One interesting implication of a possible coupling between depth and apparent sunset is that it would create an initial link between ecological and reproductive divergence in mass-spawning species. For example, a genetic propensity for successful colonization of shallower-water habitats would simultaneously reduce temporal overlap in spawning between shallow- and deeper-water forms, thus facilitating speciation. Once gene flow was reduced, the evolution of other genetic differences (including response to spawning cues) would also be possible.

The fact that the three taxa exhibit temporal differences in spawning under similar laboratory conditions suggests that the differences among them in spawning times are at least partly under genetic control. Szmant

et al. (1997) provide evidence that even intraspecific differences among colonies may have a genetic component, a conclusion supported by our occasional observations of simultaneous spawning by clonemates treated identically but distributed among several buckets.

Compatibility and selfing

Although our experimental matings were limited in number, they revealed a clear pattern that parallels results from elsewhere (Szmant et al. 1997; D. Hagman et al. in preparation). The potential for routine natural hybridization appears to be limited to crosses between *Montastraea annularis* and *M. franksi*, which is what one would expect based on the patterns of genetic differences among the three species as assayed using protein electrophoresis (Nei's *D* of 0.06 to 0.07 between *M. annularis* and *M. franksi* compared to 0.13 to 0.26 between *M. faveolata* and the other two species; Knowlton et al. 1992; Van Veghel and Bak 1993; Weil and Knowlton 1994). It remains unclear whether the failure of interspecific crosses involving *M. faveolata* is due to inefficient fertilization, early developmental abnormalities, or a combination of these two factors. It should also be emphasized that rigorous demonstration of the lack of post-zygotic isolating mechanisms between *M. annularis* and *M. franksi* would require F2 crossings, which are not practical given the long generation times of these organisms.

The failure of most selfed matings is similar to that observed for mass-spawning acroporids and other corals with buoyant gametes (Heyward and Babcock 1986; Wallace and Willis 1994), and is consistent with patterns observed in other marine invertebrates with wide dispersal abilities (Knowlton and Jackson 1993). The absence of selfing facilitates comparisons of intra- versus interspecific matings in these species, given that gametes cannot be separated before spawning.

Taxonomic implications

The results reported here add to the list of characteristics that discriminate among the three species across a wide geographic range. The broader the suite of concordant characters that differentiate taxa, the greater is the likelihood that reproductive barriers exist between them (Avise and Ball 1990). Moreover, the patterns observed suggest likely mechanisms underlying the maintenance of species boundaries.

The case for the distinctiveness of *Montastraea faveolata* is now compelling. Both the difficulty of producing viable larvae from interspecific crosses involving this species [present study; D. Hagman et al. (in preparation); and to a lesser extent Szmant et al. (1997)] and the marked allozyme differences that characterize *M. faveolata* (Knowlton et al. 1992; Van Veghel and Bak 1993; Weil and Knowlton 1994) strongly argue against routine hybridization in the field. The production of hybrid larvae observed in our crosses is likely to overestimate the potential for interspecific hybridizations in the field, since gamete concentrations were high and no choice between conspecific and heterospecific gametes was provided.

Temporal separation may play a critical role in isolating *Montastraea annularis* from *M. franksi*, which otherwise appear to be completely cross-compatible. The significance of subtle temporal differences in spawning times has generally been downplayed in species which successfully interbreed in artificial crosses, because gametes are known to be viable for several hours (Oliver and Babcock 1992; Wallace and Willis 1994). However, observations of discrete or temporally changing slicks associated with distinct pulses of spawning in the Texas Flower Gardens (Gittings et al. 1992; D. Hagman et al. in preparation) suggest that a difference of even 1 to 2 h in spawning time could isolate *M. annularis* and *M. franksi*. On the Great Barrier Reef, only gamete slicks contained enough sperm to produce high fertilization rates several hours after spawning, and these slicks tended to move off the reef within 1 h of spawning (Oliver and Babcock 1992). Thus, if sperm over the reef from *M. franksi* are too dilute to fertilize eggs spawned by *M. annularis* several hours later, and gamete slicks of the two taxa do not intermingle before *M. franksi* gametes become inviable, then the temporal barrier between the two species may be quite effective.

To determine whether these species hybridize at detectable frequencies under natural circumstances, it will be necessary to develop molecular techniques with greater resolution than protein electrophoresis. Preliminary analyses of DNA isolated from gametes collected during these spawning events suggest that amplified fragment-length polymorphisms may provide the needed level of resolution (Lopez and Knowlton 1997). Field studies of the effect of differences in spawning times on the probability of interspecific fertilization are also needed.

It will be important to measure hybridization rates at a variety of sites. Common colony morphologies in the Flower Gardens (D. Hagman et al. in preparation), the Florida keys (Szmant et al. 1997) and the Bahamas (Weil and Knowlton 1994 personal observations) appear to differ from those of Central America, with apparent intermediates being more common at the northern sites. One possible explanation is that genetic or environmental differences between sites result in characters that are problematic for species definitions based primarily on Panamanian corals. Alternatively or additionally, barriers to hybridization may be reduced in these regions, a possibility supported by the greater success of interspecific crosses involving *Montastraea faveolata* in Florida (Szmant et al. 1997).

Acknowledgements We thank S. Dementiev, G. Jácome, J. Lopez, G. Matute, F. Rodríguez and R. Tapia for assistance in the field; S. Gittings and D. Levitan for comments on an earlier version of our manuscript; D. Hagman and A. Szmant for sharing drafts of

their manuscripts; the Smithsonian Institution and the Honduras Coral Reef Fund for financial support; and the governments of Panamá (INRENARE), Honduras, and the Kuna Nation for permissions for field work and collecting.

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