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GENETIC, SPATIAL, AND TEMPORAL COMPONENTS OF PRECISE SPAWNING SYNCHRONY IN REEF BUILDING CORALS OF THE *MONTASTRAEA ANNULARIS* SPECIES COMPLEX

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When organisms release gametes into the sea, synchrony must be precise to increase fertilization and decrease hybridization. We tagged and genotyped over 400 spawning corals from the three species in the *Montastraea annularis* species complex. We report on the influence of species, individuals, and genotypes on timing of spawning from 2002 through 2009. During their annual spawning event *M. franksi* spawns on average 2 h after sunset, whereas *M. annularis* and *M. faveolata* spawn 3.5 h after sunset. Only *M. franksi* and *M. annularis* have compatible gametes. Individual colonies of the same genotype spawn at approximately the same time after sunset within and across years (within minutes), but different genotypes have significantly different spawning times. Neighboring colonies, regardless of genotype, spawn more synchronously than individuals spaced further apart. At a given distance, clone-mates spawn more synchronously than nonclone-mates. A transplant experiment indicates a genetic and environmental influence on spawn time. There is strong, but not absolute, concordance between spawn time, morphology, and genetics. Tight precision in spawning is achieved via a combination of external cues, genetic precision, and perhaps conspecific signaling. These mechanisms are likely to influence reproductive success and reproductive isolation in a density-dependent manner.

KEY WORDS: **Hybridization, reproductive isolation, reproductive success, spawning behavior, speciation.**

The timing of reproduction can influence the likelihood of fertilization, put constraints on which individuals mate with each other, affect patterns of offspring survival and dispersal, and determine the strength of reproductive isolation across species (Morgan 1995; Levitan et al. 2004; Levitan 2005). The appropriate scale for understanding the degree of synchrony in reproduction depends on the interval over which successful mating can occur. For species with internal fertilization, this interval depends on the length of time eggs are viable for fertilization and on how long females can store viable sperm or pollen. These intervals are typically in the

range of days to weeks (e.g., crabs, Paul and Adams 1984; birds, Malecki and Martin 2002; humans, Keulers et al. 2007), although in some cases receptive females are ephemeral and available for only hours (e.g., marine midges, Soong et al. 2006). In contrast, reproductive success in external fertilizers (broadcast spawning) may be extremely sensitive to even subtle differences in synchrony. In this most common marine reproductive strategy (Giese and Kanatani 1987), eggs are released into the water and may be available for fertilization for only seconds to minutes while they drift away from the spawning population. Consequences of broadcast spawning are that precise synchrony, at the scale of 10s of seconds, determines who mates with whom when sperm compete (Levitan 2005), and differences in spawn times, at the scale of 10s of minutes, can lead to reproductive isolation among sympatric populations or species (Levitan et al. 2004).

Unfortunately, we know very little about patterns of synchrony at the scale that might be important for reproductive success and reproductive isolation in broadcast spawning species. There is variation in what fraction of the population spawns during any given spawning event (Levitan 1988; Babcock et al. 1992; Oliver and Babcock 1992; Hamel and Mercier 1996; Marshall 2002) and variation in when individuals release gametes during a spawning event (e.g., invertebrates, Babcock et al. 1992; algae, Clifton 1997; corals, Levitan et al. 2004). Across different spawning events, there are some data supporting the idea that large-scale patterns of synchrony are important; on the evenings leading up to and following peak spawning nights, fewer individuals spawn, and fertilization success is proportionately reduced (Oliver and Babcock 1992; Lasker et al. 1996; Levitan et al. 2004). However, a critical gap in our knowledge is whether particular individuals always spawn during peak or off-peak times, which would make specific genotypes more or less successful or could lead to reproductive isolation.

We know even less about smaller scale asynchronies (variation in spawning times within a spawning event) and how subtle timing differences influence reproductive success. Spawning events can last for hours at the population level, but individuals often release gametes for only a fraction of this time (e.g., invertebrates, Babcock et al. 1992; corals, Levitan et al. 2004). Males often spawn before females in species with separate sexes (reviewed in Levitan 1998) and may be driven to do so by sperm competition (Levitan 2005). However, variation in spawning times exists within a sex (invertebrates, Babcock et al. 1992; algae, Clifton 1997) and also for hermaphroditic species that release gamete bundles containing both eggs and sperm (van Veghel 1994; Knowlton et al. 1997; Szmant et al. 1997; Hagman et al. 1998; Sanchez et al. 1999; Levitan et al. 2004; Wolstenholme 2004).

It is also not clear if asynchrony is a constraint imposed by rate differences in the process of gametogenesis and spawning, a result of variation in the timing of when individuals receive spawning cues, or is an adaptive response. Evidence for sex differences in spawning and the proposed adaptive explanations for these behaviors (Levitan 2005; Lotterhos and Levitan 2010), as well as the notion of disruptive selection on spawning time to avoid polyspermy (Tomaiuolo et al. 2007), suggest that at least some fraction of asynchrony could have adaptive explanations. However, there is also evidence that achieving precision in spawning can be difficult when the process of gametogenesis takes months (Fadlallah 1982; Soong 1991). Variance in any of the steps leading up to spawning, including gametogenesis, gamete packaging, cue reception, and gamete release can accumulate and result in asynchronous spawning among individuals in a population and thereby constrain reproductive success.

Determining the evolutionary consequences of variation in spawning time requires repeated measures of the timing of spawning within and among genotypes while ideally accounting for environmental factors. To this end, we have established a long-term monitoring and experimental study of spawning times in corals. Many coral species only spawn on a very few predictable evenings each year (Harrison et al. 1984; Babcock et al. 1986; Szmant 1986; van Veghel 1994; Hagman et al. 1998). This predictability in when corals spawn and their sessile lifestyle make replicated observations of spawning of individuals possible at very fine scales. In addition, because these corals can fragment, we can compare and manipulate multiple fragments (i.e., ramets) of the same genotype (i.e., genet). This allows examination of variation in spawning times within individuals and genotypes and variation in the degree of synchrony from year to year during annual spawning events. The results presented here suggest that individual corals are remarkably precise year after year and that there is a genetic component to this precision, but that the degree of precision is influenced by a variety of intrinsic and extrinsic factors that are likely to have important consequences in determining reproductive success and reproductive isolation.

STUDY SPECIES

Of all the Caribbean corals that engage in mass spawning, reproduction in the *Montastraea annularis* complex is the best studied (Szmant 1991; van Veghel 1994; van Veghel and Bak 1994; van Veghel and Kahmann 1994; Knowlton et al. 1997; Szmant et al. 1997; Hagman et al. 1998; Levitan et al. 2004). Corals in this group are hermaphroditic and have external fertilization. Most polyps in a colony produce a gamete bundle that contains both sperm and eggs. These bundles are constructed before spawning and become obvious about 30 min prior to spawning as the bundle works its way through the pharynx of the polyp (termed "setting"). After release, the gamete bundles float slowly to the surface (van Veghel 1994). As they approach and reach the surface, they break apart, releasing the eggs and sperm into the water column. The eggs are positively buoyant, but the sperm are neutrally buoyant.

Each colony releases a set of gamete bundles within a few minutes, and most conspecific colonies that spawn on a particular evening release gamete bundles within 30–60 min of each other (Knowlton et al. 1997; Szmant et al. 1997; Hagman et al. 1998; Levitan et al. 2004).

All three taxa in this species complex—*M. annularis*, *M. faveolata* and *M. franksi*—spawn four to eight days after the full moon in late August, September, or early October, with timing during this period dependent on latitude (Gittings et al. 1992; Knowlton et al. 1997; Szmant et al. 1997; Levitan et al. 2004). Spawning at a location can be split among two or perhaps all three months, with mid-September being the peak time in Panama. *Montastraea* spawns one month earlier in Bermuda and often so in Florida (Wyers et al. 1991; Szmant et al. 1997) and one month later in Curaçao (van Veghel 1994), perhaps due to local differences in the annual temperature cycle (van Veghel 1994), solar irradiance (van Woesik et al. 2006), or wind patterns (van Woesik 2010).

Fertilization assays in Panama and the Bahamas indicate that *M. faveolata* gametes are largely incompatible with *M. franksi* and *M. annularis* (Levitan et al. 2004). The rare exceptions are as infrequent as successful self-crosses and not statistically different from 0% fertilization. The other two congeners are largely compatible at these same sites. These patterns of compatibility are consistent with data from other Caribbean regions (reviewed in Levitan et al. 2004).

Spawning times for these species are remarkably consistent, within an evening, across years, and regions. The mean spawn time for *M. franksi* is 115 min after sunset, whereas *M. annularis* and *M. faveolata* have mean spawn times of 225 and 235 min after sunset, respectively, in both Panama and the Bahamas (Levitan et al. 2004). Each individual colony releases its gametes within approximately 1 min, and each species spawns within approximately 1 h (Levitan et al. 2004). Thus there is a gap in spawning between the early spawning *M. franksi* and the later two species. These patterns are consistent throughout the Caribbean as noted in other studies (Colombia, Curacao, Florida, Gulf of Mexico, Honduras—reviewed in Levitan et al. 2004). The cue for spawning time appears to be sunset; regional differences in spawning times can be explained by adjusting for local sunset time, and corals can be induced to spawn early by placing the corals in darkness prior to sunset (Knowlton et al. 1997; Levitan et al. 2004; Brady et al. 2009).

Gametically compatible *M. franksi* and *M. annularis* are the most likely congeners to produce hybrid larvae. However, given their differences in spawning times, hybrid fertilization may be rare. The obstacles to hybrid fertilization include gamete aging, gamete dispersal, and gamete dilution (Levitan et al. 2004). In addition, these species are often found in different habitats (Weil and Knowlton 1994). Sperm age faster than eggs, and the early spawning *M. franksi* produces sperm that are much less effective at fertilizing eggs after 2 h. Field studies measuring the fertilization potential of *M. franksi* sperm during spawning events indicate that sperm reach the water's surface and become effective at fertilizing eggs approximately 30 min after spawning and lose this ability 60–90 min later, when sperm dilution and aging make fertilization unlikely (Levitan et al. 2004). Thus sperm from *M. franksi* lose their ability to fertilize eggs just before *M. annularis* eggs become available. The more likely scenario for hybrid fertilization depends on sperm limitation, resulting in *M. franksi* eggs remaining unfertilized by conspecifics until they are exposed to freshly spawned *M. annularis* sperm later in the evening. Although we often measure sperm-limited conditions during these spawning events, these eggs often drift hundreds to thousands of meters during the interval between the *M. franksi* and *M. annularis* spawn (Levitan et al. 2004). The likelihood of this cross depends on the spatial extent of reefs, the location of various species, and water flow. These constraints on the likelihood of hybrid fertilization may explain why there are regional differences in the degree of genetic similarity (and the implied level of introgression) between these two species (Fukami et al. 2004).

Methods

FIELD SITE

We have established a permanent site for monitoring coral spawning at Bocas del Toro, Panama (9″19′38N, 82″12′14W). The reef is protected on all sides by islands and follows a 3–8 m depth contour off Solarte Island. The monitored section of reef is approximately 100 m along this depth contour and 30 m wide, and is composed primarily of *M. franksi* and *M. annularis* (Fig. 1A). Between 2002 and 2009, monitoring generally started on the third day following the full moon in September and continued until at least 7 days following the full moon. Between 6 and 12 divers, wearing synchronized watches, patrolled the site starting at 1945 h (approximately 75 min after sunset, 15 min before the first setting colony and 30 min before the first spawning *M. franksi* colony) until 2230 h (approximately 30 min after the last spawning *M. annularis* colony). Nine subsurface buoys mark the site, set at approximately 8-m intervals. The line of these buoys is lit by using green chemical lights (glow sticks) to orient divers. Two-person dive teams monitor a subset of the site that overlaps with other dive teams for a full coverage of the site. Set and/or spawn times are recorded to the minute, along with coral tag numbers, for all observed corals setting or actually spawning. For all untagged corals (corals never seen to spawn previously), and a subset of tagged corals (for orientation purposes) a red glow stick attached to a lead fishing weight via a 10-cm nylon line is activated and placed on the setting or spawning coral. The glow sticks are numbered, as is a 10-cm strip of flagging tape attached to the nylon line.

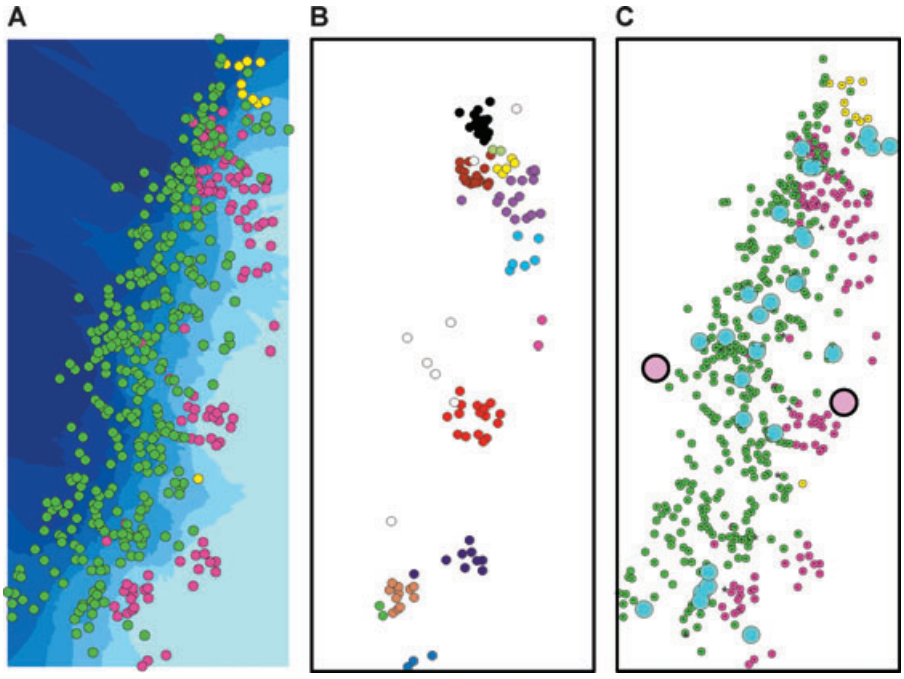

Figure 1. Map of study site in Bocas del Toro, Panama. (A) Location of *M. franksi* (green), *M. annularis* (magenta), and *M. faveolata* (yellow) individual colonies. The lightest shade of blue indicates 2.5 m of water depth, each darker shade is 0.5 m deeper (max 8 m). Map area is 78 × 36 m. (B) Clonal structure of *M. annularis* colonies. Each color represents a different genotype, white colonies represent genotypes with only one ramet. (C). Location of "late spawning "*M. franksi* colonies" (large teal circles) and transplant experiment (large pink circles).

During the day following a night of spawning, these glow sticks are replaced with permanent numbered aluminum tags, the corals' positions are mapped, top and bottom of the colony depth recorded, and tissue samples are taken for genetic analysis. In the spring of 2008, N. Knowlton, blinded to spawning times, recorded morphological traits and binned corals into species. In the fall of 2008, the height, longest diameter of each coral colony, and its perpendicular diameter was recorded and the percent live tissue was estimated for *M. franksi* and *M. faveolata* corals (*M. annularis* corals were often discontinuous columns of tissue and this method would not accurately reflect living coral size). Surface area of coral colonies was estimated from the height, length, and width of each colony estimated as an elliptical hemisphere.

MONTASTRAEA SAMPLING AND DNA EXTRACTION

Montastraea tissue plugs containing four to five polyps were collected by using a small leather punch (approximately 1.5 cm diameter) and digested and preserved in CHAOS [4M guanidine thiocyanate, 0.1% N-lauroyl sarcosin sodium, 10 mM Tris pH8, 0.1 M 2-mercaptoethanol (Fukami et al. 2004)]. The tissue samples in CHAOS were left at room temperature for at least 72 h then stored at −20°C until extraction. DNA extractions were performed by mixing 50 μl of digested coral tissue, 10 μl Sprintprep Activator (magnetic beads from Beckman Coulter Genomics/Agencourt Bioscience Corporation Davens, MA), and 80 μl of 100% isopropanol. Subsequently, samples were placed on a magnetic plate for 10 min, then drained and rinsed with cold 70% EtOH. Rinsing was repeated five times, and samples were allowed to dry for 60–90 min before resuspending in 50 μl of 1 × TE buffer and placing on a shaker table for 1 h. The DNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to 5 ng/μl using sterile double distilled water and then stored at −20°C until ready for use in PCR reactions.

GENOTYPING

Six microsatellite loci from Severance et al. (2004) were used for genotyping. The PCR cocktail consisted of 2.4 μl 5X PCR buffer (Promega, Madison, WI), 1.2 μl 1mM dNTPs, 0.15 μl GoTaq (Promega, Madison, WI), 1.0 μl 10 μM bovine serum albumin, 1.25–3.5 μl 1.5 mM $MgCl_2$ (depending on primer), 0.5 μl of fluorescently labeled forward primer, 0.5 μl reverse primer, 2.0 μl DNA (5 ng/μl), and double distilled water to bring to a total volume of 12 μl. PCR amplification was run as follows: 95°C for 3 min, then 30 cycles of 95°C for 1 min, 50°C (primers maMS11, maMS2–4) or 55°C (primers maMS8, maMS12, maMS2–5, and maMS2–8), 72°C for 2 min, then a final extension time of 30 min at 72°C. PCR product from three loci with different fluorescent labels were multiplexed (mulitplexI: maMS8, 2–4, 2–5 and multiplexII: maMS11, 12, 2–8) using HiDI Formamide (1:12) and 0.5 μl Genescan 400 ROX (Applied Biosystems, Foster City, CA) and analyzed with

Applied Biosystems 3130×l Genetic Analyzer with Capillary Electrophoresis (Foster City, CA). Finally, Genemapper software (Applied Biosystems, version 4, Foster City, CA) was used to check for misidentified peaks and stutter bands. All alleles were binned into di- or tri-nucleotide sizes dependent on locus. Individuals with ambiguous genotypes were rerun to confirm binning. All loci were analyzed with MICRO-CHECKER (Oosterhout et al. 2004) to calculate the observed and expected heterozygosity, the likelihood of large allele drop-out and null alleles.

To examine colonies as a function of whether they were independent genetic individuals or a product of asexual reproduction, these six microsatellite loci were used to assign colonies into genets. The probability that individuals sharing the same genotype were generated by sexual reproduction (as opposed to clone-mates) was estimated by GIMLET (Valiere 2002) as being $<3 \times 10^{-10}$, and so we assume that individuals that share a genotype are the products of asexual reproduction. Occasionally, a set of spatially related *M. annularis* individuals would share the same genotypes at all loci but one. In such cases, one allele would match, whereas the other allele would have a one bin shift (two or three nucleotides depending on the repeat size). To test for PCR artifacts in these cases, we routinely reamplified these individuals and found identical results. To further test if these cases were amplification errors, we chose four colonies (two typical genotypes and two with the bin shift) from each of four spatially distinct genets (16 colonies) and reextracted (two replicates) and reamplified (two replicates per extraction) the DNA. In every case all reruns confirmed the original assignment (five amplifications from three extractions per colony); spatially defined genets were often comprised of intermingled individuals with a one allele, one bin shift. Because of the unlikely nature of one bin shifts being spatial neighbors, and because even siblings would not be expected to show one bin shifts in relatedness, we classified these as somatic mutations and pooled these nearly identical genotypes into the same genet.

TRANSPLANT EXPERIMENT

In 2007, we conducted a transplant experiment to examine the influence of genotype and location on spawning times in *M. annularis*. Corals were collected 1 km from the study site, at a location with a similar depth profile and distribution of corals. This collection site has discontinuous colonies of *M. annularis*. Three ripe colonies found at a similar depth of approximately 4–6 m but separated by at least 50 m were selected, and six columns from each colony were collected. For each colony, each column was approximately 15 cm in diameter, and all columns from a colony were connected by a continuous tissue before collection. Three columns (ramets) from each colony (genet) were placed in one of two locations. One location was in 2.5 m of water in the *M. annularis* zone and the other was in the downstream direction (on nights of coral spawn) 30 m west in 8 m of water in the *M. franksi* zone (locations noted on Fig. 1C). Within each location, individual ramets were placed approximately 10 cm apart in a 3×3 Latin square. Once setting was noted in these corals, a diver was assigned to each location to record spawning times.

POPULATION STRUCTURE

Montastraea annularis and *M. franksi* are recently diverged taxa and have been shown to have significant genetic differences in some (e.g., Panama) but not all (e.g., Bahamas) locations in the Caribbean (Fukami et al. 2004). Even within Panama, the available markers have not revealed diagnostic loci (Fukami et al. 2004). This makes it difficult to distinguish potential hybrids from rare parental genotypes. We used our microsatellite data to estimate population structure of *M. annularis* and *M. franksi* individuals and also to consider individual assignment into populations in light of the spawning time for each genotype. We only used a single individual from each genet in cases where there were multiple ramets per genet. The program MICRO-CHECKER (Oosterhout et al. 2004)) was used to check microsatellite loci for null alleles and large allele drop-out. The program STRUCTURE version 2.3.2 (Pritchard et al. 2000) was used to estimate the number of populations and also the probability of assignment into each population. We conducted the analysis in two modes: first using "admixture" (which did not use morphology as a prior), and second using "loc prior" (which used morphology instead of location as a prior). The use of these priors has been shown not to influence inferring structure when no structure exists (Hubisz et al. 2009). Because these are different species, we assumed independent allele frequencies for each species and ran three replicate runs for each of seven values of the assumed number of populations ($K =$ 1–7). We used a burn in of 50,000 iterations followed by 50,000 Markov chain Monte Carlo runs.

We confirmed STRUCTURE results using the program NewHybrids version 1.1 (Anderson and Thompson 2002) to specifically address the probability of individuals with ambiguous assignments being pure species or of mixed ancestry. NewHybrids allows identification of specific individuals as belonging to a particular species and then assigns, for a class of questionable individuals, the probability of being a pure species or having mixed ancestry. We used a 100,000 iteration burn in followed by an additional 300,000 iterations in three replicate runs.

Results

We observed, tagged and genotyped 488 spawning corals at our study site for a total of 1335 observations of setting or spawning from 2002 through 2009. Of these colonies, based on morphological features, 361 were *M. franksi*, 117 were *M. annularis* and 10 were *M. faveolata* (Fig. 1A). The most repeat observations on individual corals were from colonies tagged in 2002, the first year

of observation. *Montastraea franksi* corals tagged in 2002 were observed to spawn an average of 5.95 times and *M. annularis* 5.25 times over the eight years of this study. Corals tagged in later years had correspondingly fewer observations. Larger *M. franksi* corals were also noted to spawn more frequently than smaller corals. Larger corals also tended to be tagged in earlier years. However, a multiple regression indicated that both factors, the number of years the coral was tagged ($F = 159.27$, $P < 0.0001$) and the size of the coral ($F = 46.41$, $P < 0.0001$) independently influenced the number of spawning observations. We cannot be certain if these results are because large or tagged corals spawn more often or because they are more obvious when they spawn. The largest observed *M. franksi* colony spawning was 45,000 cm^2, the smallest 300 cm^2.

TEMPORAL PATTERNS OF SPAWNING WITHIN AND AMONG CORAL SPECIES

Corals spawned on the same set of evenings following the full moon in September. On the fourth day following the full moon (day of full moon equals "day 0") either zero or a small number (one to three colonies) of *M. franksi* corals were observed to spawn. Peak spawning occurred on days five and six with greatly reduced spawning by day seven (Fig. 2). Spawning was observed every year, even during 2005 when there was a widespread coral bleaching event throughout the Caribbean (Eakin et al. 2010) and some bleaching was noted in *M. annularis* colonies at our site. There was no apparent effect of what time during the day the full moon occurred (ranging from 02:01 to 18:46) on the day corals were first observed to spawn. Most corals were only observed to spawn on one evening per year, but occasionally individual corals spawned partially on consecutive nights. Individual corals did not seem to have a preference for spawning on a particular night. To test for preference, we examined the subset of spawning or setting observations in which a colony was observed to spawn on either day five or day six for four years. Chi-square analysis indicated that individuals did not significantly depart from a random distribution of no preference ($P > 0.05$); there was no evidence of within species temporal isolation by day. On the last night of spawning (day 7), few colonies spawned and most observations were of only a few polyps releasing bundles.

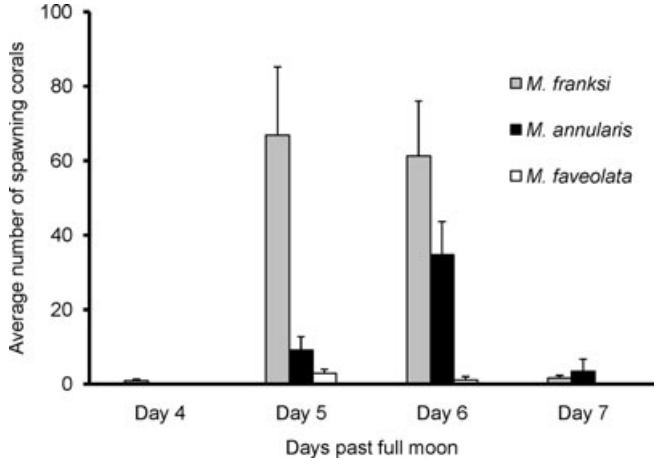

Figure 2. Distribution of spawning colonies as a function of days following the full moon. Day "0" is the day of the full moon.

An overall analysis of variance (ANOVA) examined the main effects of species, lunar day, and year on spawn time. There were clear species differences and significant two-way interactions between species and lunar day and species and year (Table 1). The mean spawning time in minutes after sunset was 117 for *M. franksi*, 209 for *M. annularis* and 231 for *M. faveolata* (Fig. 3A). Each species spawned for approximately 1 h. These means and ranges in spawn time are consistent with our earlier work in Panama and with others sites around the Caribbean (Levitan et al. 2004). Mean spawn time differs by more than 100 min between *M. franksi* and *M. annularis*, the two compatible species (Fig. 3B). Subtle yearly differences in mean species spawn time seem to apply to both species; both species shift spawning times by 5–10 min each year in concert (Fig. 3C). The notable exception, likely driving the interaction between species and year, was in 2006 when *M. annularis* spawned approximately 30 min later than typical and was not mirrored by delayed spawning in *M. franksi*. At present, we have not correlated these yearly shifts with environmental factors and they remain unexplained. Individuals of *M. franksi* and *M. faveolata* spawned a few minutes later on lunar day six compared to day five, whereas *M. annularis* tended to spawn earlier on day six compared to day five. These differences are greater than can be accounted for by differences in sunset times across days but less than can be accounted for by differences in moon rise across days. These species differences in lunar day shifts in spawning likely account for the species by lunar day interaction. Because of significant interactions, each species was examined independently testing the main effects of lunar day, year, and individual colony on spawning time (Table 1). For *M. annularis* and *M. franksi* all main effects were significant and the interaction between lunar day and year was significant (Table 1). *Montastraea faveolata*, with greatly reduced sample size, demonstrated significant year and lunar effects but not individual colony effects on spawn time (Table 1).

It is particularly interesting that there was a consistent occurrence of a second, smaller peak in spawning of 27 individuals morphologically classified as *M. franksi*. This second peak consisted of 7% of the *M. franski* individuals and spawning in this group occurred 140–160 min after sunset, approximately 40 min after the major peak in *M. franksi* and 60 min before the peak *M. annularis* spawn (Fig. 3A). There was a gap of approximately 10 min between the last individual from the major peak to the first individual in the minor peak in *M. franksi* spawning. We have noticed this secondary peak in our second permanent site off of

Table 1. Fixed effects model ANOVA of spawn time as a function of species, lunar day, year, and two-way interactions. The three-way interaction was first included in the model, but was found not to be significant and was consequently removed. Subsequent ANOVAs used a mixed model to test each species independently with the same fixed effects and also included individual colony as a random factor.

Source	df	Type III SS	MS	*F*	*P*
Overall test					
Spp	2	0.07656	0.03828	271.95	<0.0001
Lunar	3	0.00127	0.00042	3.01	0.0295
Year	7	0.00706	0.00100	7.17	<0.0001
Spp×Lunar	3	0.00186	0.00062	4.40	0.0044
Spp×Year	11	0.00576	0.00052	3.72	<0.0001
Lunar× Year	10	0.00436	0.00044	3.10	0.0007
Error	853	0.12007	0.00014		
Total	889	0.94338			
Species					
M. annularis					
Colony	98	0.01419	0.00014	1.77	0.0009
Lunar	2	0.00060	0.00030	3.68	0.0277
Year	7	0.00546	0.00078	9.52	<0.0001
Lunar×Year	5	0.00177	0.00036	4.34	0.0010
Error	144	0.01180	0.00008		
Total	256	0.03863			
M. franksi					
Colony	300	0.08345	0.00028	8.92	<0.0001
Lunar	3	0.00046	0.00015	4.89	0.0025
Year	7	0.00057	0.00008	2.60	0.0128
Lunar×Year	9	0.00073	0.00008	2.60	0.0067
Error	294	0.00916	0.00003		
Total	613	0.10439			
M. faveolata					
Colony	8	0.00051	0.00006	2.27	0.2228
Lunar	1	0.00037	0.00037	13.12	0.0223
Year	3	0.00135	0.00045	16.15	0.0106
Lunar×Year	1	0.00001	0.00001	0.54	0.5050
Error	4	0.00011	0.00003		
Total	18	0.00547			

Carrie Bow Cay, Belize (D.R. Levitan and N. Knowlton, unpubl. data). We call the individuals in this secondary peak "late spawning *M. franksi.*" The location of these individuals are not clumped, but largely spread across the 2–6 m depth contour (Fig. 1C).

There was a nearly perfect concordance of morphological species identification and spawning time for *M. franksi* and *M. annularis*, the two sister taxa (Weil and Knowlton 1994) with compatible gametes (Levitan et al. 2004). No colonies morphologically assigned to *M. annularis* spawned earlier during the *M. franksi* period. Of the 361 *M. franksi* colonies spawning for a total of 614 times, only three colonies were noted to spawn later in the evening during the *M. annularis* period. Because we have no a priori expectation that these corals were unique in any way, we included them in the analysis as *M. franksi* individuals. All three of these colonies were relatively small and nestled underneath larger *M. annularis* colonies and were only observed to spawn on the first year they were tagged (i.e., before a permanent tag was in place). There are three possible explanations for these late spawning individuals. They might represent the tail in the distribution of *M. franksi* spawning that overlaps with *M. annularis*, they might be *M. annularis* colonies that are morphologically similar to *M. franksi*, or they might have been mistakenly tagged when the larger *M. annularis* colonies spawned next to them. Statistical analyses excluding these individuals did not vary qualitatively when they were eliminated from the analysis (not shown).

PREDICTABILITY OF INDIVIDUALS SPAWNING ACROSS YEARS

Individual corals spawned at approximately the same time each year. The average standard deviation in spawn time for a particular

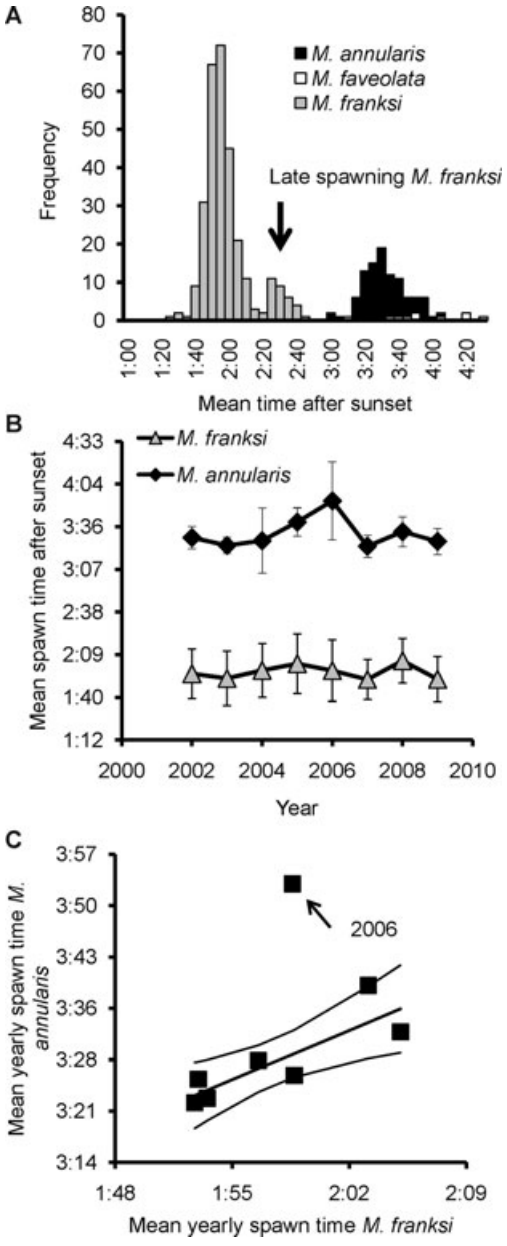

Figure 3. Mean spawn time for corals across years. (A) Frequency distribution of mean spawn time for each colony. The secondary smaller peak of *M. franksi* corals is highlighted with an arrow. (B) Yearly differences in spawn time for *M. franksi* and *M. annularis*. Species mean and standard error in spawn time each year. (C) Species mean spawn time for *M. annularis* as a function of mean spawn time for *M. franksi*. Upper and lower bounds of 95% CI of means is calculated without 2006; in that year *M. annularis* spawned later in relation to *M. franksi* compared to all other years (labeled outlier point).

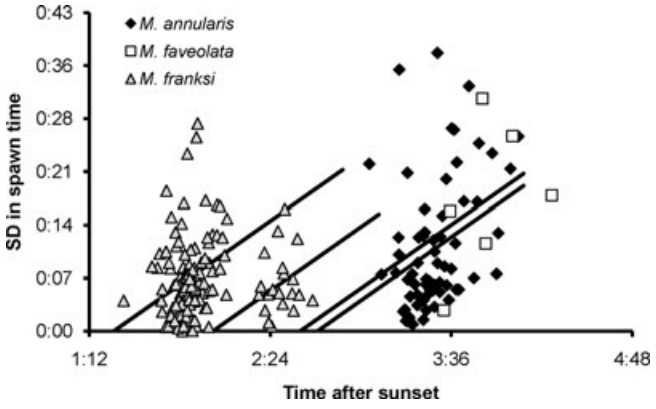

Figure 4. Standard deviation in spawn time for each coral colony across years as a function of that coral's mean spawn time after sunset. ANCOVA did not indicate significantly different slopes for each spawning group (early and late spawning *M. franksi*, *M. annularis* and *M. faveolata*), but significantly different intercepts. Slope and intercepts calculated by general linear model (Table 2).

colony was 7 min for *M. franksi*, 10 min for *M. annularis,* and 14 min for *M. faveolata*. The average standard deviation for the late spawning *M. franksi* individuals across years was 6 min. These individuals consistently spawned in the gap between the majority of early spawning *M. franksi* individuals and the later spawning *M. annularis*. The intraindividual variation in spawn time is related to when the corals typically spawned after sunset. Both within and among species, corals that spawn later in the evening were less precise from year to year (Fig. 4). Corals may lose some ability to spawn precisely as the time from the cue increases. To test for variability in precision, we used an analysis of covariance (ANCOVA) with the main effect of species and the covariate of mean spawn time for each colony and used the standard deviation in spawn time for that colony (over time) as the response variable. For curiosity, we assigned the late spawning *M. franksi* as a separate group. We first tested for significant differences in slopes (interaction of main effect with covariate), found none, and removed the interaction from the model. We noted a significant main effect (group) and covariate (mean spawn time); for each group the variance in spawn time increased with time after sunset (Table 2). Pairwise tests of the least square means indicate that early spawning *M. franksi*, late spawning *M. franksi,* and *M. annularis* all have significantly different intercepts. This suggests that the later spawning *M. franksi*, like the later spawning *M. annularis* and *M. faveolata*, appears to be responding to the sunset cue independently. These late spawning *M. franksi* individuals tend to have an intermediate depth distribution, compared to the earlier spawning *M. franksi* individuals and the later spawning *M. annularis* individuals. However, there is a wide overlap among these species in depth distribution (Fig. 5).

TEMPORAL PATTERN OF SPAWNING WITHIN AND AMONG GENETS

Montastraea annularis, which typically inhabited shallower water (more wave action) and has a columnar morphology (more likely to fragment—Foster et al. 2007), had 21 genetic individuals among the 117 sampled colonies (Fig. 1B). All colonies

Table 2. ANCOVA testing the influence of mean spawn time of a colony on its standard deviation in spawn time across years. The covariate was the mean spawn time for each colony, the main effect was the species or spawn group. For this analysis, *M. franksi* corals were split into early and late spawning groups. There was no significant interaction of spawn time and species ($P = 0.84$), and this term was removed from the model. The standard deviation in spawn time increases with mean spawn time past sunset. Each species or group has the same positive slope (Fig. 5).

Source	df	Type III SS	MS	*F*	*P*
Species or groups	3	0.000408	0.000136	6.81	0.0002
Mean spawn time	1	0.000443	0.000443	22.19	<0.0001
Error	204	0.004075	0.000020		
Total	208	0.005164			

sharing the same genotype were spatially related (neighbors or near neighbors) and were often found on the same reef structure (a small patch surrounded by sand). In contrast, *M. franksi* rarely fragmented. We genotyped 351 *M. franksi* corals and 324 of those were unique genotypes. Only 19 genetic individuals had asexually propagated colonies (comprised of two to four spatially adjacent ramets each); over 90% of *M. franksi* corals on this reef are the product of sexual reproduction. Although relatively rare at this site, *M. faveolata* had an intermediate level of fragmentation; the 10 colonies sorted into three spatially arranged genets. During the 2008 spawning event, two to three gamete bundles were collected from each of two *M. faveolata* colonies into a 30-mL syringe in a series of pairwise crosses within and across these two genets. Crosses within presumed genets failed to fertilize, whereas crosses between presumed genets did result in fertilization. Previous data (Levitan et al. 2004) established that these corals have blocks to self fertilization. Thus genetic data matched the expectations established by gametic compatibility. These three genets also consistently differed in color.

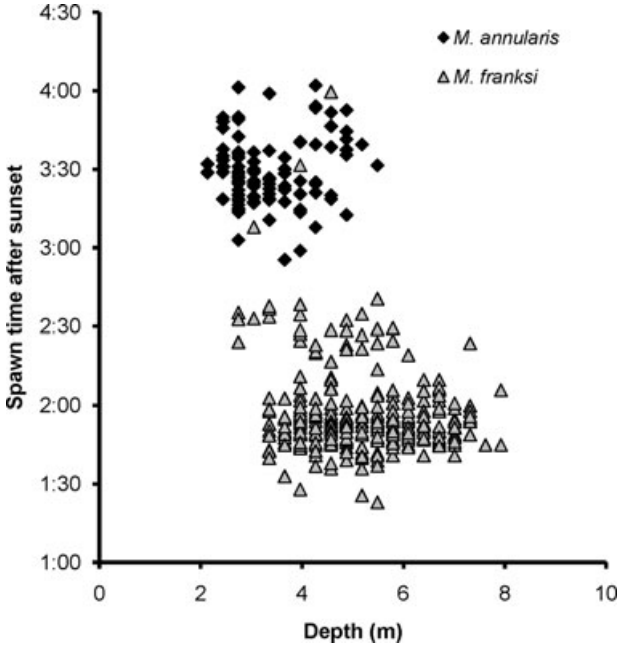

Figure 5. Spawn time of each colony as a function of depth for *M. franksi* and *M. annularis*.

All three species were tested independently to examine whether individuals within a genet and different genets had significant differences in spawn time. This test only included those genets containing multiple colonies ($n = 17$ genets of *M. franksi* had multiple ramets, 11 *M. annularis* and two *M. faveolata*). An mixed model ANOVA testing for the effect of genet, individual colony nested within genet, lunar day and year on spawn time found a significant effect of genet, but no significant effect of individual colony nested within genet for *M. annularis* and *M. franksi* (*M. faveolata* with reduced sample size had a marginally nonsignificant effect of genet, Table 3); individuals within a genotype did not differ in spawn time, whereas different genetic individuals had, overall, significantly different spawn times (Fig. 6). Typically each species spawned for a period of around 1 h, whereas corals within a genet spawned within several minutes. The early spawning species, *M. franski*, showed no significant effect of year or lunar day on spawn time. The later spawning *M. annularis* and *M. faveolata* did show differences in spawn time across years and lunar day. This effect is largely driven by 2006 data in which these species spawned approximately 30 min later than typical. *Montastraea annularis* also demonstrated significant interactions between these time effects and genet. *Montastraea faveolata*, because of its rareness at this site, did not have the sample size to allow for tests of interactions. Although these interactions are potentially interesting, the variance within a genotype across years, lunar days, and individual colonies is slight compared to the differences among genotypes (Fig. 6).

SPATIAL PATTERNS OF SPAWNING

We used the SPATSTAT (Baddeley and Turner 2005) modules written for R to estimate all pairwise distances among colonies. Within each species (*M. annularis* and *M. franksi*), pairwise differences in the mean spawn time of each coral was compared with their pairwise spatial distances in a Mantel test. These analyses were conducted at the ramet level (all colonies within a species)

Table 3. Nested mixed model ANOVA testing the effects of genet (random effect), colony nested within genet (random effect), year (fixed effect), and lunar day (fixed effect) on spawn time. Each species was tested independently. Nonsignificant interactions were removed from the models testing *M. annularis* and *M. franksi*. Samples sizes were not adequate to test for interactions with *M. faveolata*.

Source	df	III SS	MS	*F*	*P*
M. annularis					
Genet	10	0.00209	0.000209	2.53	0.0113
Error	72.59	0.00601	0.000083		
ID (Genet)	76	0.00322	0.000042	1.03	0.4431
Lunar	2	0.00095	0.000477	11.57	<0.0001
Genet×Year	38	0.00533	0.000140	3.41	<0.0001
Genet×Lunar	7	0.00118	0.000169	4.09	0.0006
Year×Lunar	3	0.00034	0.000114	2.76	0.0468
Error	92	0.00379	0.000041		
Year	7	0.00191	0.000274	3.19	0.0057
Error	66.51	0.00571	0.000085		
M. franksi					
Genet	17	0.011105	0.000653	25.60	<0.0001
Error	29.60	0.000755	0.000026		
ID (Genet)	23	0.000057	0.000025	0.80	0.7107
Year	7	0.000267	0.000038	1.23	0.3142
Lunar	2	0.000131	0.000066	2.10	0.1367
Error	36	0.001121	0.000031		
M. faveolata					
Genet	1	0.000183	0.000183	3.65	0.0862
Error	9.64	0.000483	0.000050		
ID (Genet)	7	0.000440	0.000062	2.48	0.1671
Year	3	0.001345	0.000448	17.68	0.0043
Lunar	1	0.000426	0.000433	16.81	0.0094
Error	5	0.000127	0.000025		

and at the genet level (mean spatial position and spawn time of each genetic individual). At the ramet level, for both species, there was a significant relation between the distance between colonies and their difference in spawn time; closer colonies spawned in greater synchrony ($P = 0.0002$ for *M. franksi* and 0.003 for *M. annularis*, Fig. 7A,B). This effect reached an asymptote at around 5 m. When colonies were greater than 5 m apart they spawned at random with respect to each other. At the genet level, this relationship was nearly identical in *M. franksi* ($P = 0.0002$), almost certainly because this species generally did not fragment and thus the analysis was nearly identical. The pattern was the same with *M. annularis*, but likely because of reduced sample size (only 18 genets) the relationship was not significant. This analysis indicates that neighbors, whether they are clone-mates or unrelated individuals, tend to spawn more synchronously than individuals spaced further apart.

Because clone-mates tend to be in close proximity (Fig. 1B), the tight synchrony of clone-mates (Fig. 6) could be caused by genetic similarity or simply reflect neighbor effects demonstrated above. We attempted to tease apart these confounding effects with *M. annularis*, the only species with robust samples sizes of clone-mates. We sorted all pairwise data as either clone-mate or nonclone-mate pairs and only examined the data where the distribution of pairwise distances among these two classes were similar. This range was chosen by eliminating pairs that were closer than 4.5 m (95% of nonclone-mates had greater distances) and further than 6.2 m (95% of clone-mates had smaller distances). This left 75 pairs of clone-mates and 272 pairs of nonclone-mates with a similar distance distribution (Fig. 7C). Over this range, clone-mates spawned more synchronously (mean = 9 min, median = 4 min) than nonclone-mates (mean = 15 min, median = 13 min).

To test for the spawn difference between clone-mates versus nonclone-mates while accounting for distance over this subset of data, we compared the actual data to a null distribution estimated from randomizations. We bootstrapped 10,000 replicates of a null distribution by shuffling the genotypes and recalculating relatedness, then estimating the 95% confidence intervals on the randomized slopes (distance) and intercepts (spawn difference) for clone-mates and nonclone-mates. This analysis found

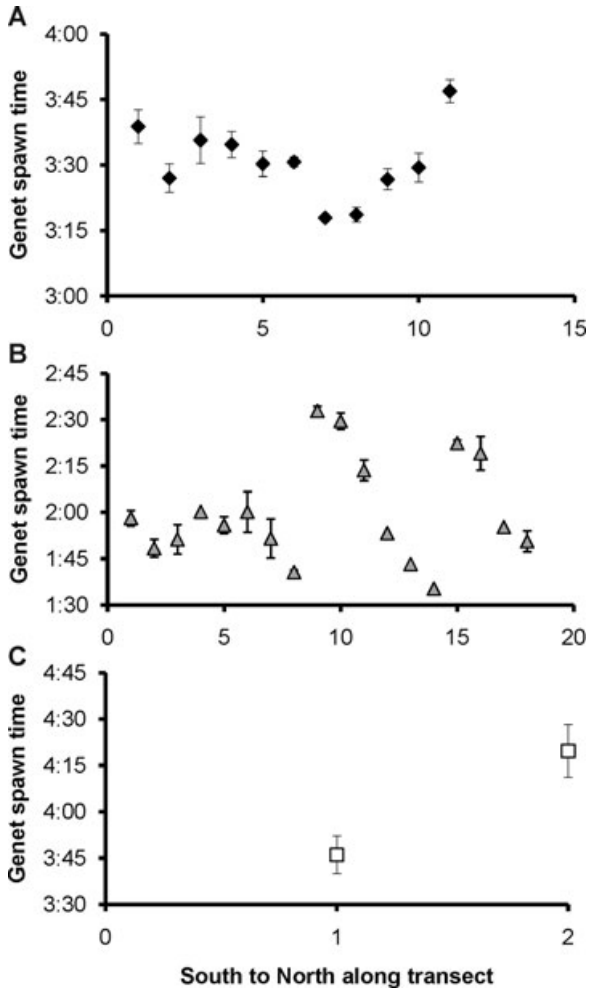

Figure 6. **Spawn time after sunset within and across each genotype. The *x*-axis is ranked sequence along the transect with one symbol per genet. Only genets with multiple ramets were included. Standard error in spawn time includes differences among ramets and evenings. (A) *M. franksi*, (B) *M. annularis*, (C) *M. faveolata*.**

no significant affect of distance over this short range, and so we then performed the same randomization tests on the mean spawn-difference between clone-mates versus nonclone-mates. The difference between the actual means in spawn time difference among pairs of clone-mates compared to pairs of nonclone-mates (6 min) is significantly greater than the 95% confidence intervals from the randomized null distribution (2 min). Thus clone-mates spawn more synchronously than nonclone-mates at similar neighbor distances.

TRANSPLANT EXPERIMENT

All three genotypes spawned on the same evening, and spawning times were recorded for 16 of the 18 ramets. A random effects two-way ANOVA testing the effect of genotype and location on spawn time noted significance in both effects ($P < 0.0001$ for location and $P = 0.02$ for genotype) and no significant interaction. The variance components of the ANOVA model indicate that 82% of the variance in spawn time was explained by location whereas 8% was explained by genotype. The same rank order of spawn times among the three genets was noted in both locations, but the corals at the deeper site, the site further from the *M. annularis* zone, was phase shifted 14 min later (Fig. 8). Genetic differences remained across locations, but location was a stronger influence on spawn time.

POPULATION STRUCTURE

MICRO-CHECKER (Oosterhout et al. 2004) indicated that two loci consistently had significant excess of homozygotes, in both *M. annularis* and *M. franksi*, likely explained by the presence of null alleles (Table 4). These two loci also showed excess homozygosity in the initial study describing these microsatellite loci (Severance et al. 2004). All analyses were conducted twice; with and without these two loci. The STRUCTURE version 2.3.2 (Pritchard et al. 2000) analysis using morphology as a prior indicated strong support for two populations (($P > 0.9999$, calculated as the $\Pr(X/K_2)/\Sigma\Pr(X/K_{1-n})$ as in Pritchard et al. (2000)) compared to all others populations tested (K 1, 3–7 all $P < 0.00001$) when either all six or only four loci were considered. When morphology was not used as a prior the admixture results were less clear on the number of populations. Using all six loci the Ln Pr(X/K) reached an asymptote at $K = 4$, whereas runs using only four loci gave strongest support for a single population. In all of these admixture runs the pattern was to assign the majority of the *M. annularis* individuals to one population and then approximately divide the *M. franksi* individuals among all populations. Patterns of assignment into the two populations ($K = 2$) as a function of morphology and spawn time are shown for both sets of assumptions (Fig. 9A,B). The results suggest distinctive assignment of *M. annularis* individuals and a wider range of assignments for *M. franksi* individuals. This, in part, may be caused by the much larger sample of *M. franksi* genotypes compared to *M. annularis*. Both of these STRUCTURE models suggest that many, but not all, of the 27 late spawning *M. franksi* individuals are genetically more similar to *M. annularis*. The admixture model indicates that some of these late spawning *M. franksi* individuals are firmly assigned to the *M. annularis* population (Fig. 9A), while the model that uses morphology as a prior suggests that this subset has a more intermediate assignment (Fig. 9B). Regardless of which model is used, a large fraction of the individuals that spawn between the early spawning *M. franksi* and the late spawning *M. annularis* is morphologically similar to *M. franksi* and genetically more similar to *M. annularis*. The remainder of this fraction appears to be morphologically and genetically similar to *M. franksi*. It is also worth noting that the three corals identified as *M. franksi* that were reported to spawn once late in the evening with *M. annularis* are genetically firmly within the *M. franksi* population (Fig. 9B).

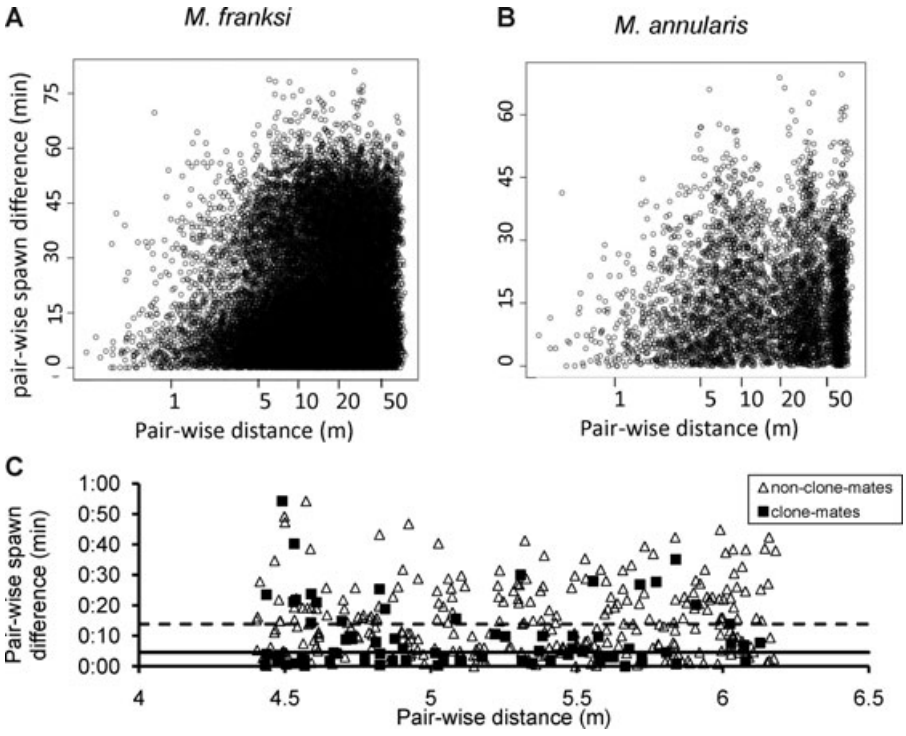

Figure 7. **Pairwise difference in spawn time (minutes) as a function of pairwise distance between colonies (m) for *M. franksi* (A) and *M. annularis* (B). Nearby colonies spawn synchronously, whereas colonies located ca 5 m apart spawn at random with respect to each other. (C) Subset of *M. annularis* data over the range with overlapping values for pairwise distances between clone-mates (black squares) and nonclone-mates (open triangles). Horizontal lines are median spawn time differences for clone-mates (solid) and nonclone-mates (dotted). At this distance clone-mates spawn more synchronously than nonclone-mates.**

The program NewHybrids version 1.1 (Anderson and Thompson 2002) was used to specifically examine the genetic status of individuals classified morphologically as the late spawning *M. franksi* corals. We assigned the early spawning *M. franksi* and late spawning *M. annularis* as pure species and used NewHybrids to assign the 27 intermediate spawning individuals (late spawning *M. franksi*). Three replicate runs were conducted using all six loci and also by excluding the two loci with excess homozygosity (Table 4). The results from using six or four loci were subtly different, but with the same general interpretation. Using all six loci, the analysis of the "late spawning *M. franksi* individuals" indicated that in all three replicate runs, all but two of the individuals were assigned to one species or the other at a probability more than 90% (Fig. 9C). These 25 individuals were more often assigned to *M. annularis* (59% compared to 30% to *M. franksi*) and there was no pattern of assignment associated with spawn time within this group. The two individuals with lower likelihoods of assignments had, in all three replicate runs, a 61% and 26% chance of having mixed ancestry, with the largest fraction of that probability always being an F1 hybrid. When the two loci with homozygote excess were removed from the analysis, all individuals were assigned to one species or the other with a probability more than 90% (and all but one with a probability more than 98%); there was no evidence for mixed ancestry in any individual (results not shown). Both sets of analysis provide little to no support for intermediate spawning individuals being hybrids or backcrosses between *M. annularis* and *M. franksi*. It is also worth noting that while the STRUCTURE analyses often produced low probabilities of assignment into the two species, the NewHybrid analysis, which did not use morphological priors on these most ambiguous individuals, assigned individuals to one

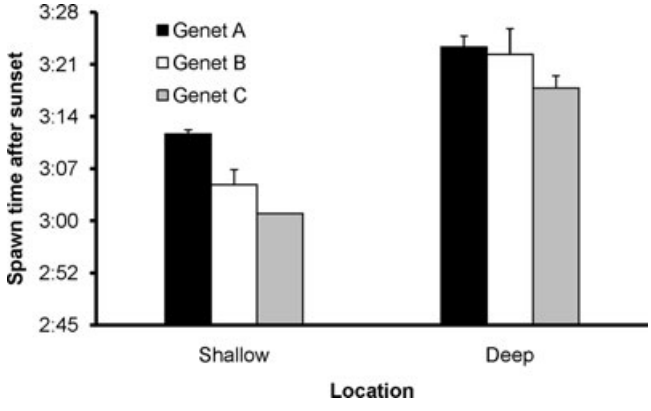

Figure 8. **Spawn time in transplant experiment. Each genet was split into six ramets, three ramets of each genet placed in one of two locations. There was a significant effect of genotype and location but no significant interaction.**

Table 4. **Characteristics of microsatellites loci used in analysis. Details on primer sequences can be found in Severance et al. 2004. Only one representative individual analyzed for genets with more than one ramet (clone-mates). Data presented independently for *M. annularis* and the early and late spawning *M. franksi* individuals. Numbers in parentheses are numbers of individuals. Data for *M. faveolata* not presented as they only represented three genets. For each species, the number of individuals, number of alleles, observed and expected levels of heterozygosity, probability of significant heterozygosity deficiency (<0.05 or 0.0001), and the estimated frequency of null alleles (MICRO-CHECKER, Oosterhout et al. 2004) are calculated. There was no evidence for large allele drop-out.**

Locus	No. of alleles	He ob	He exp	*P*	null
M. annularis (22)					
maMS11	19	0.591	0.921	***	0.180
maMS2-8	9	0.636	0.825	*	0.111
maMS2-4	9	0.864	0.728		−0.121
maMS12	14	0.524	0.896	***	0.205
maMS2-5	13	0.773	0.862		0.056
maMS8	3	0.273	0.280		0.047
Early *M. franksi* (296)					
maMS11	44	0.507	0.942	***	0.231
maMS2-8	21	0.801	0.878		0.036
maMS2-4	15	0.780	0.810		0.018
maMS12	29	0.510	0.881	***	0.207
maMS2-5	19	0.901	0.919		0.010
maMS8	4	0.651	0.641		−0.010
Late *M. franksi* (30)					
maMS11	18	0.552	0.906	***	0.194
maMS2-8	13	0.733	0.884		0.085
maMS2-4	12	0.931	0.863		−0.040
maMS12	16	0.500	0.872	***	0.211
maMS2-5	14	0.933	0.870		−0.041
maMS8	3	0.367	0.406		0.067

or the other species with a high probability. In sum, the small handful of intermediate spawning corals appears, genetically, to be a mixed group of individuals from one or the other species, but morphologically more similar to *M. franksi*.

Discussion

Corals in the *M. annularis* species complex spawn predictably in Panama on the fifth and sixth nights after the full moon in September. On our monitored reef, we observed over 400 coral colonies spawning over a total of 1300 times. We did not observe all corals spawning every year and cannot make clear statements about whether a coral that was not observed to spawn did or did not spawn. We do have accurate records suggesting that individuals and individuals nested within genotypes spawn at remarkably similar times year after year. Although each species spawns for well over an hour, individuals have a characteristic spawn time that typically varies over 10 min or less. This variance within an individual includes lunar and yearly differences that shift the entire species earlier or later on a particular evening. Further, individuals with the same genotype do not significantly differ in spawn times, although different genotypes do differ. These genotypic differences in spawn time appear to be modulated by the environment. A spatial analysis of spawn time indicate that neighbors, whether they were the same or different genotypes, spawned synchronously, whereas colonies spaced further than 5 m apart spawned at random with respect to each other. Superimposed on this neighbor effect, clone-mates spawned more synchronously than nonclone-mates at distances where both types of pairwise comparisons were common. These genetic and environmental patterns were supported by a transplant experiment. The rank order of spawning times among genets remained the same across transplanted locations, but the absolute spawning times shifted from one location to the other.

These patterns suggest a hierarchy of spawning cues (reviewed in Baird et al. 2009). Previous research has suggested that patterns of solar irradiance and/or wind fields cues the month (van Woesik et al. 2006; van Woesik 2010), the lunar cycle cues the day (Richmond and Jokiel 1984; Levy et al. 2007), and sunset cues the time of spawning (Knowlton et al. 1997; Levitan et al. 2004; Brady et al. 2009). Our work indicates that there appears to be random or unknown factors that determine which lunar day a coral spawns (e.g., night 5 or 6), but the timing on any given night is a function of how genetic and local environmental factors interact with the sunset cue. Two lines of evidence point to

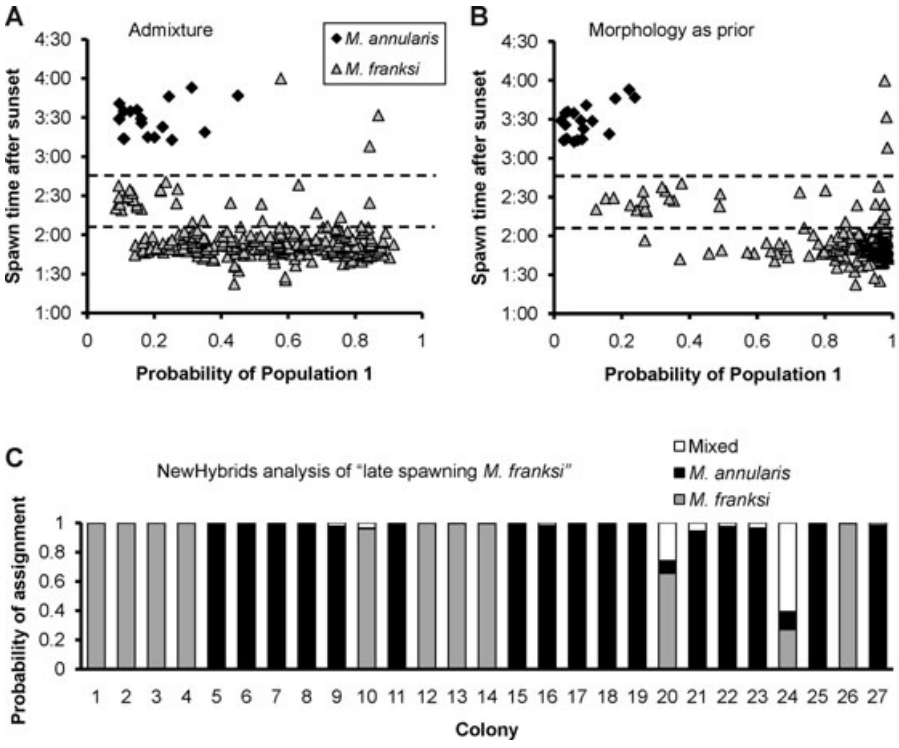

Figure 9. **Genetic analysis using STRUCTURE and NewHybrids. (A) STRUCTURE analysis using admixture model with independent allele frequencies, $K = 2$ populations. The probability of assignment into population no. 1 is plotted as a function of spawn time after sunset. Black diamond symbols represent individuals morphologically assigned as *M. annularis*, gray triangles as *M. franksi*, dotted line represents lower and upper bound to "late spawning *M. franksi* individuals". (B) STRUCTURE analysis using morphology as a prior (LocPrior) with independent allele frequencies, $K = 2$ populations. Symbols the same as panel A. (C) NewHybrids analysis of the probability of assignment of the 27 "late spawning *M. franksi* individuals" into *M. annularis* (black bars), *M. franksi* (gray bars), or mixed ancestry (combined probability of F1, F2, or backcrossed—white bars). Colonies are arranged from early to late spawning from left to right. Morphology is used as a prior on the two "source" populations (the early spawning *M. franksi* and the later spawning *M. annularis*) but not on these 27 individuals that spawn at intermediate times. The probability of mixed ancestry in these individuals is almost always less than 5%, and most individuals are strongly assigned to one pure species or the other. All figures generated using six loci.**

sunset as an important cue. First, artificially induced early sunsets (coral shading) result in earlier spawning of corals (Knowlton et al. 1997; Levitan et al. 2004; Brady et al. 2009). Second, here we note that the variance in spawning time within a colony across years increases as a function of mean spawn time after sunset. Although variance is often related to the magnitude of the mean, this only reinforces the point; a particular time of day only has a magnitude when it has a reference to another time. In this case, the reference appears to be the sunset cue for spawning. The relatively steep slope of this relationship suggests that if the cue were much earlier in the day, or if spawning was delayed another few hours into the evening, the precision of corals might break down to the point where spawning was asynchronous. This may explain why spawning in many taxa occurs shortly after sunrise or sunset.

The environmental cue responsible for neighbor synchrony could be a spawning pheromone or some microhabitat differences across the reef. Although we cannot entirely rule out microhabitat differences, it does not appear to be depth related, and there do not appear to be temperature-related gradients within a depth contour (unpublished data from temperature loggers set at 8-m intervals at 4-m depth). In addition, although there is some support for deeper corals spawning earlier from a previous study conducted over a larger depth gradient (Levitan et al. 2004), the transplant experiment showed the opposite trend; the corals placed deeper (but further from conspecifics) delayed spawning compared to corals placed next to conspecifics in shallower water. There is evidence that soft and hard corals release hormones into the sea during spawning events (Atkinson and Atkinson 1992; Slattery et al. 1999; Terrant et al. 1999; Twan et al. 2006). Although there have not been experimental tests to determine if these hormones induce conspecifics to spawn, it does provide evidence that such chemicals are available in the water to act as a synchronizing agent. Our working hypothesis is that sunset may trigger setting times in corals and eventually these corals will release these gamete bundles (as evident when corals are isolated in the laboratory, Levitan et al. 2004), but that they may release these bundles sooner and more synchronously with neighbors as the local threshold of pheromone concentration is reached for a patch of corals. The

delayed spawning of the transplanted corals 15 m downstream of the conspecific population compared to the transplants within the conspecific population could be explained by chemical cues arriving later or not arriving in sufficient concentrations to elicit earlier spawning.

Comparing fertilization success on peak versus off-peak evenings of spawning and across reefs with different densities indicates that fertilization success increases with an increased number of spawning colonies (Levitan et al. 2004). In addition, within an evening, corals that spawn at peak times during the spawning event have higher fertilization compared to individuals that spawn even 15 min earlier or later than the mean (Levitan et al. 2004). The possibility that neighbors might increase synchrony via pheromones suggests that there may be two consequences of low densities on reproductive success in corals. The first would be a direct effect of a reduction in the number and distance of individuals releasing sperm, which would lead to reduced sperm concentrations and lower fertilization success. The second is an indirect effect in that if spawning pheromones become less effective at lower densities or greater distances between conspecifics, then these fewer spawning colonies might also be less synchronous. Both these direct and indirect factors might increase the likelihood of Allee effects in depleted coral populations. There has been a world-wide reduction in coral abundances, particularly of these large, long-lived, broadcast spawning species (Hughes 1994; Gardner et al. 2003). Recent surveys have listed these *Montastraea* species as being either endangered or vulnerable (Carpenter et al. 2008). Reductions in densities might reduce per capita reproductive success and hinder or prevent recovery of these populations, even if the factors responsible for these declining populations were alleviated.

The data presented here indicate that colonies spawn consistently at particular times after sunset, and that there is a genetic component to this timing. This should result in an evolutionary response imposed by this stabilizing selection to spawn during peak times. Increased synchrony can be achieved when organisms use a series of cues (Soong et al. 2006). As individuals wait for the next cue, any rate differences in gametogenesis or gamete bundle formation accumulated since the last cue will be eliminated or dampened. Although some exceptionally fast or slow developers might miss one particular cue, they will be binned with other rapid or slow individuals into the next spawning period. This process may explain why different cohorts of individuals spawn on the same lunar day on different months, or different evenings on the same lunar cycle; individuals progressing too fast or slow are binned into different spawning events. This process can explain the random process of which day a particular coral spawns, but a highly predicable and genetically influenced time of spawning post sunset; the particulars of how resource availability or stress influencing the rate of gametogenesis and gamete packaging may differ from year to year or month to month, but once individuals are binned into a particular day they will spawn at a time, in part determined by genotype. These individuals will have a chance at fertilization in proportion to the number of other individuals that are similarly binned for that day.

Theory has suggested that sperm availability can influence the evolution of spawning times and result in temporal reproductive isolation in sympatry (Tomaiuolo et al. 2007). This model characterized how sperm overabundance can lead to polyspermy and disruptive selection on spawning time. Because spawning time variation can lead to assortative mating within a time period, the two prerequisites for sympatric speciation, disruptive selection and assortative mating (Dieckmann and Dobelli 1999; Gavrilets 2004; Burger et al. 2006), are satisfied with only one trait. This model may be relevant on reefs of high coral densities and offer a potential explanation for how closely related and co-occurring species speciated in times past. This scenario is unlikely to apply to current reefs, as most reefs have suffered moderate-to-severe reductions in coral abundance (Hughes 1994; Gardner et al. 2003). However the corollary of this hypothesis is that at times of lower densities, selection will favor stabilizing selection for increased synchrony that might result in the eventual reticulation of coral species for which subtle temporal barriers may be the only mechanisms preventing hybridization. The rate at which corals might adapt spawning times to changed environmental conditions will depend on the strength of selection and the amount of standing genetic variation. The current analysis suggests that, although there is a genetic component to spawning, much of the variation in spawn time, within species, is attributed to environmental factors (e.g., yearly, lunar and local environment). This should not be all that surprising given the large selective disadvantage to spawning even slightly out of synchrony (Levitan et al. 2004) that would tend to eliminate genetic variants that spawn off peak times.

Detecting hybridization in the *M. annularis* species complex has been problematic because these corals, in particular *M. annularis* and *M. franksi*, are genetically very similar (Fukami et al. 2004) to the point where diagnostic alleles are lacking that could distinguish between an individual having a rare genotype for a particular species versus being a hybrid or backcrossed individual. Although our genetic analysis would benefit from having additional informative loci, these two species show strong concordance between morphology, spawn time, and genetics, with an interesting caveat. The early spawning species, *M. franksi*, has a small secondary peak in spawning at the end of the *M. franksi* spawning period and before the *M. annularis* period. These corals also tend to have an intermediate depth distribution between these coral species. Our genetic analysis suggests that this group of individuals is more likely a mixture of pure individuals from both species, rather than being hybrid individuals. Further investigation might reveal some level of introgression that could explain the

fascinating pattern that some of these individuals are morphologically similar to *M. franksi*, but genetically similar to *M. annularis*. In addition these intermediate spawners appear to respond the sunset cue as an independent group, suggesting they share some trait that determines the response time to sunset. Phenotypic plasticity might play some role, as they are found at intermediate depths, however, *M. annularis* and *M. franksi* have overlapping depth distributions at this site and for the vast majority of individuals that spawn at the "proper" time, morphology and genetics are all concordant with spawn time, regardless of depth. Evidence of limited genetic introgression in other coral taxa support the notion that introgressed genes that influence morphology, and other functional traits, might be selected out of backcrossed individuals (Vollmer and Palumbi 2002). Although our data, and previous work (Fukami et al. 2004), provides little evidence for introgression at this site, there may be regional differences in the degree of introgression across these species (Fukami et al. 2004). Previous research on reproductive success as a function of synchrony suggests that hybrids formation is unlikely and that individuals with intermediate spawn times would have reduced reproductive success (Levitan et al. 2004). The intermediate spawners at our site, regardless of their genetic identity, are likely to have reduced fitness as they are more likely to spawn in isolation.

In conclusion, individual coral genotypes have very predictable spawning times that are likely to produce predictable fitness consequences for individuals that spawn during the peak or tails of spawning events. Under conditions of sperm limitation, which is often observed in these corals, corals spawning in off peak times have greatly reduced reproductive success (Levitan et al. 2004). This combination should result in strong stabilizing selection and the evolution of sharp spawning peaks. That neighbors also spawn in synchrony will further increase the likelihood of fertilization and that close conspecific neighbors are the most likely individuals to mate. This combination of genetic and environmental factors will not only increase fertilization rates within species but also reproductive isolation across species.

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