Reproductive and Genetic Evidence for a Reticulate Evolutionary History of Mass-Spawning Corals

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Reef-building corals, which reproduce through simultaneous multispecies spawning, are thought to hybridize frequently, and it is hypothesized that they have evolved in repeated rounds of species separation and fusion. We conducted cross-fertilization experiments and molecular analyses with a number of mass-spawning coral species in the genus *Acropora.* A high rate of interspecific fertilization occurred between some species despite very different morphologies. The hybrid larvae developed normally and contained an allelic sequence transmitted from each parent, suggesting common diploid hybridization. Molecular phylogenetic analyses provided strong evidence for a gene pool shared between the hybridizing species. These reproductive and genetic characteristics are consistent with a species complex formed under the separation/fusion processes predicted for a reticulate evolutionary history.

Introduction

Many reef-building corals reproduce sexually in a unique synchronous ''mass spawning.'' In the Indo-Pacific region, mass spawning occurs once a year, on a night in early summer, near time of a full moon (Harrison et al. 1984; Babcock et al. 1986; Hayashibara et al. 1993). Huge quantities of eggs and sperm are released simultaneously by vast numbers of coral colonies belonging to many species and genera. The gametes congregate on the surface of the water over the reef to form ''slicks'' up to a few kilometers long. Although the mechanisms are still unknown, synchronized spawning promotes the fertilization of gametes released by sedentary colonies within each species and decreases mortality from predation pressure. Mass spawning, however, makes it inherently difficult to preserve species identities. Since many species and genera growing side by side spawn simultaneously, fertilization may occur between related species and produce a significant number of hybrids, unless eggs and sperm have an effective mechanism for recognition and fertilization only within their own species. This process is particularly important for the genus *Acropora,* which dominates Indo-Pacific reefs and contains a large number of species with a variety of morphologies.

Based on the presence of fossils and colonies with intermediate morphologies and the successful fertilization in the laboratory of eggs with sperm collected from different species (Wallace and Willis 1994; Willis et al. 1994), it is suggested that hybrid formation occurs in this genus during mass spawning. Based on this type of evidence, Veron (1995) applied the hypothesis of ''reticulate evolution'' to mass-spawning corals (see also Grigg 1995). According to this hypothesis, current coral species did not evolve from a common ancestral species through the continual separation of new species along discrete lineages. Instead, they evolved through repeated rounds of species separation and fusion, forming a reticulate pattern of many interconnecting lineages (fig. 1). An important feature of this hypothesis is that multiple species are involved in the separation and fusion processes. If an existing group of species is currently undergoing separation, the species involved may still be connected reproductively and share common DNA polymorphisms, although they have developed species identities. In reverse, if an existing group of species is undergoing fusion, the species involved may hybridize to some extent and share a common gene pool through the exchange of genes (gene introgression) via hybrids. We tested these possibilities by conducting simultaneous DNA analyses and crossing experiments for *Acropora* species of various morphologies.

Materials and Methods

Species Identification and Collection of Specimens

All specimens were collected from reefs around Akajima Island, Okinawa, Japan (30°N, 123°E). Species identification was based on Veron and Wallace (1984) and Veron (1986). The species used are shown in figure 2. *Acropora nasuta* forms azalea-bush-shaped colonies. *Acropora digitifera* has similar morphologies, but this species develops a thick base layer. *Acropora formosa* and *Acropora nobilis,* both forming dendritic colonies, are distinguishable from each other by differences in the shapes of the corallites, small cuplike structures along the branches holding individual polyps. *Acropora florida* forms bottlebrushlike colonies with irregular branching. These species, which grow sympatrically, often side by side, can be identified by consistent morphological characters even if they are collected from different environments.

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Key words: *Acropora,* coral, gene introgression, hybridization, mass spawning, reticulate evolution.

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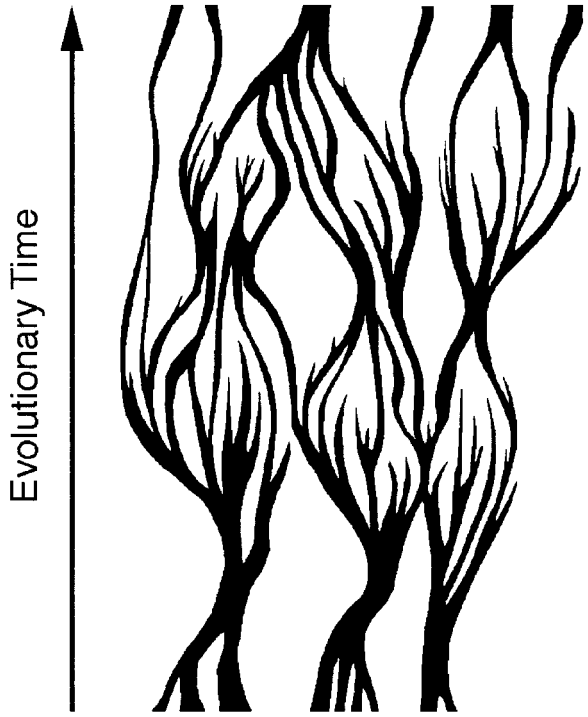

FIG. 1.—Concept diagram of reticulate evolution (modified from Veron 1995).

Crossing Experiments

Colonies were collected and kept in the sea until just before spawning, at which time they were transferred into individual buckets. Gamete bundles were then collected from individual colonies and allowed to break apart in a small volume of filtrated seawater to yield free eggs and sperm suspensions. The experiments that followed were done in a room kept at about 25°C. Eggs were washed twice with filtrated seawater, and sperm suspensions were diluted to adjust their concentrations. Eggs and sperm collected from individual colonies were then mixed in pairwise combinations. One hundred to five hundred eggs were mixed with sperm in 50-ml vials within 4 h after spawning; within that period fertilization efficiency was kept at a high rate (Wallace and Willis 1994). Sperm concentration was 1×10^5/ml, at which effective fertilization was reported for *Acropora* (Willis et al. 1994). The numbers of fertilized and unfertilized eggs were scored at the morula/blastula stage 4–8 h after the mixing of gametes.

Metamorphosis Induction

The embryos obtained were transferred to new vials filled with filtrated seawater and allowed to develop to planula larvae for 7–10 days. Ten larvae for each batch were transferred to 10- ml cups, and algal chips were added to induce metamorphosis as described in Morse et al. (1996).

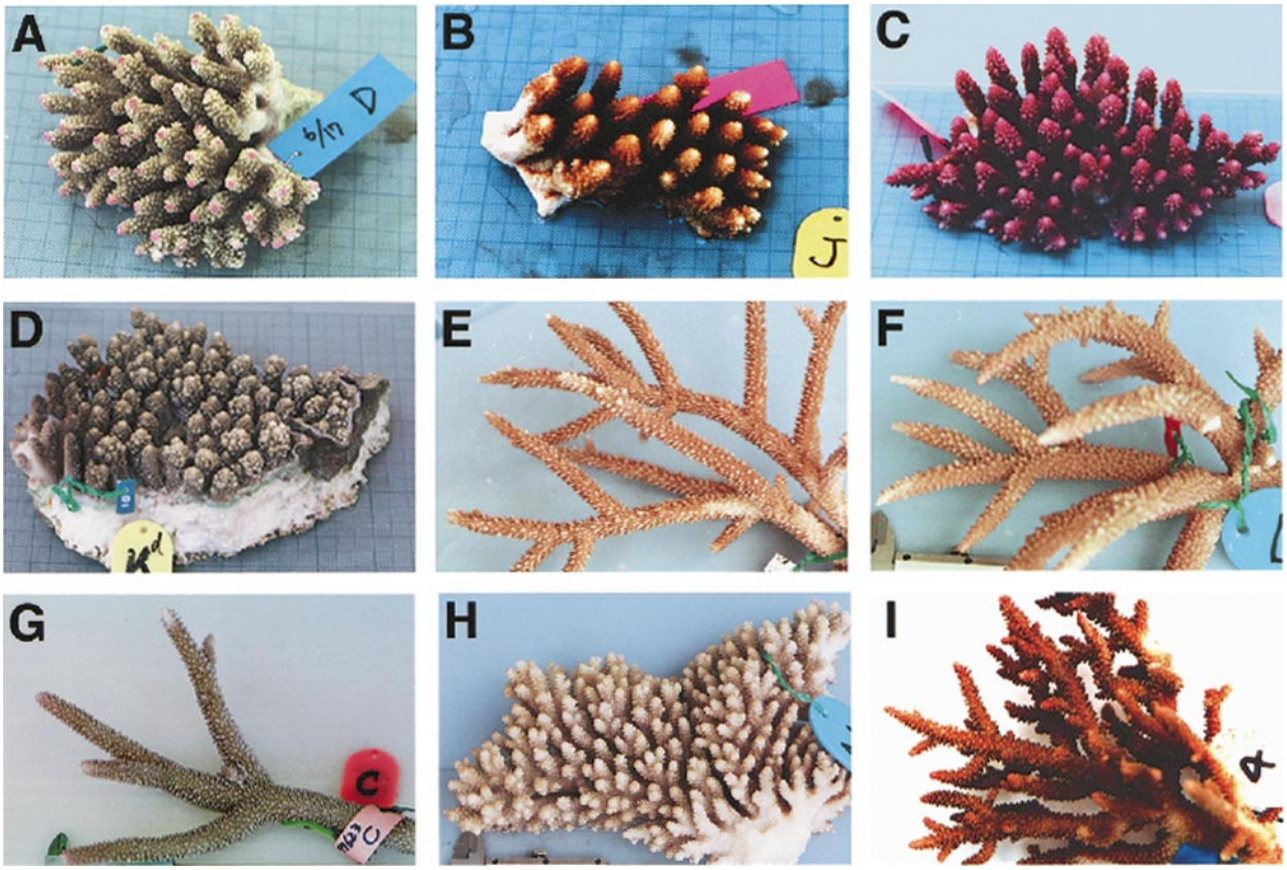

FIG. 2.—Representative colony types of the genus *Acropora*. *A, Acropora nasuta*-A; *B, Acropora nasuta*-B; *C, Acropora nasuta*-C; *D, Acropora digitifera*; *E, Acropora formosa*-A; *F, Acropora formosa*-B; *G, Acropora nobilis*; *H, Acropora florida*; *I, Acropora donei.*

Table 1
Fertilization Within and Between *Acropora* Species

	Sperm							
Egg	*A. nasuta*-A	*A. nasuta*-B	*A. nasuta*-C	*A. formosa*-A	*A. formosa*-B	*A. digitifera*	*A. nobilis*	*A. florida*
A. nasuta-A	90.0 ± 24.6 (40/53)	0.0 ± 0.2 (0/27)	1.1 ± 3.1 (0/9)	45.1 ± 31.1 (17/19)	0.2 ± 0.4 (0/11)	3.6 ± 20.5 (3/43)	0.2 ± 0.4 (0/5)	0.1 ± 0.2 (0/20)
A. nasuta-B	0.5 ± 1.7 (0/21)	90.6 ± 22.1 (18/18)	0.1 ± 0.3 (0/10)	0.0 ± 0.0 (0/9)	NT	0.3 ± 0.8 (0/19)	NT	NT
A. nasuta-C	96.8 ± 2.5 (9/9)	0.3 ± 0.6 (0/11)	96.0 ± 1.0 (2/2)	94.5 ± 1.5 (2/2)	NT	0.0 ± 0.0 (0/7)	NT	NT
A. formosa-A . . .	76.5 ± 26.8 (12/12)	0.5 ± 0.9 (0/4)	40.0 ± 27.0 (2/2)	83.5 ± 16.5 (2/2)	1.6 ± 1.5 (0/5)	12.6 ± 15.8 (2/6)	0.0 ± 0.0 (0/2)	1.0 ± 0.0 (0/1)
A. formosa-B . . .	0.5 ± 0.8 (0/12)	NT	NT	1.2 ± 1.5 (0/5)	71.0 ± 33.7 (5/6)	0.7 ± 0.1 (0/6)	0.0 ± 0.0 (0/4)	1.0 ± 0.8 (0/3)
A. digitifera	0.2 ± 0.9 (0/43)	0.0 ± 0.0 (0/17)	14.3 ± 32.1 (1/6)	0.0 ± 0.0 (0/12)	0.0 ± 0.0 (0/6)	98.4 ± 1.9 (22/22)	0.0 ± 0.0 (0/2)	0.0 ± 0.0 (0/2)
A. nobilis	0.0 ± 0.0 (0/5)	NT	NT	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/4)	100.0 ± 0.0 (2/2)	0.0 ± 0.0 (0/1)
A. florida	0.0 ± 0.0 (0/5)	NT	NT	0.0 ± 0.0 (0/2)	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/5)	71.5 ± 28.5 (2/2)	94.0 ± 2.2 (3/3)

Note.—Fertilization percentages of individual crosses are averaged and shown with standard deviations. Values for the intraspecific crosses are the averages of both reciprocal combinations of egg and sperm. The numbers in parentheses are the numbers of crosses which yielded >10% fertilization out of the total numbers tested. NT = not tested.

DNA Analyses

DNA was extracted by conventional methods, using proteinase K followed by phenol extraction and ethanol precipitation, from sperm collected by centrifugation of the suspension or from individual planula larvae. The second intron region of the mini- collagen gene (Wang et al. 1995) was amplified by PCR with primers 5′-TGTACTTGCATCGTGTCTTGTAGCCATAG-3′ and 5′-ATAGGTCCCATACATCCTGGTGTGCC-3′ under 30 cycles of 25 s at 94°C, 30 s at 65°C, and 45 s at 72°C using Expand HiFi polymerase (Roche). Amplified fragments were separated by agarose electrophoresis, blunted, cloned in pBluescript, and sequenced for both strands. PCR misincorporation was excluded by analyzing more than five clones obtained from one to three independent PCRs. DNA sequences ca. 250 bp long were manually aligned and analyzed with software programs ODEN (Ina 1994) and CLUSTAL W, available in DDBJ and TreeView (PHYLIP).

Results

Interspecific Fertilization

The pooled results of five separate experiments carried out during the mass spawning events of 1994–1997 are summarized in table 1. Each value in the table is the average percentage of fertilization for a pairwise cross. In this study, a ''species'' is defined as a group of individuals that is both a morphologically identified type and a reproductively connected unit. *Acropora formosa* was found to form two reproductive units. Since each reproductive unit was consistent with a morphological type formerly thought of as intraspecific variation, the reproductive units were tentatively designated independent species, *A. formosa*-A and *A. formosa*-B. Similarly, *A. nasuta* was found to consist of three separate reproductive units that corresponded with three morphological types, designated *A. nasuta*-A, *A. nasuta*-B, and *A. nasuta*-C.

Intraspecific crosses produced high average fertilization rates for all eight species, and the rates for all but a few individual crosses exceeded 90%. For example, only one out of six combinations of three colonies of *A. formosa*-B had 0% fertilization. On the other hand, in most cases, there was near 0% fertilization for self-crosses between eggs and sperm collected from the same colony, indicating the presence of a mechanism to prevent self-fertilization in acroporids. The values for self-crosses were excluded from the computations for the values in table 1.

In most cases, interspecific crosses had very low fertilization rates (<2%), suggesting general reproductive isolation at the level of fertilization between species. High rates of fertilization, however, occurred in nine interspecific crosses. The cross between *A. nasuta*-A and *A. formosa*-A, two species with distinct morphologies (fig. 2*A* and *E*), is typical. Sperm of *A. formosa*-A also fertilized 94.5% of *A. nasuta*-C eggs, while the rate of fertilization for reciprocal combination was 40.0%. Although the average percentage of fertilization was low (3.6%) between *A. nasuta*-A eggs and *A. digitifera* sperm, there was significant fertilization (12%–76%) in 3 out of 43 individual crosses. Interspecific fertilization occurred in 8 of the 12 possible pairings involving *A. formosa*-A, *A. nasuta*-A, *A. nasuta*-C, and *A. digitifera* in an essentially nonreciprocal manner. Interspecific fertilization was also observed between *A. florida* eggs and *A. nobilis* sperm. These two species also have very different morphologies (fig. 2*G* and *H*). Interspecific fertilization occurred only in specific combinations of the individual recognized species, at differing rates, while the fertilization rates within species were very high, except in a few cases.

All of the hybrid embryos were active and developed to planula larvae normally; some also metamorphosed to polyps. There was no difference in the metamorphosis frequencies of intraspecific and interspecific

Table 2
Examples of Allelic Sequence Comparison Between *Acropora* Parents and Their Larvae

Animals	Allele	Nucleotides at Positions[a]	Allele Type	Accession No.
A. nasuta-A colony E (egg donor)	1	ATTATTTCCTTTCTT-T	naA-E1	AB009724
	2	ATCATTTCCTGTCTTCC	naA-E2	AB009725
A. nasuta-C colony A (sperm donor)	1	ACTATTTCCTTTCTT-T	naC-A1	AB009729
	2	TTTATTTCCTTTCTT-T	naC-A2	AB009730
Larva-1	1	ATTATTTCCTTTCTT-T	naA-E1	
	2	TTTATTTCCTTTCTT-T	naC-A2	
Larva-2	1	ATCATTTCCTGTCTTCC	naA-E2	
	2	TTTATTTCCTTTCTT-T	naC-A2	
A. nasuta-C colony F (egg donor)	1	ATTATCTCCTGTTTT-T	naC-F1	AB009731
	2	ATTATCTTATGTTTT-T	naC-F2	AB009732
A. nasuta-A colony E (sperm donor)	1	ATTATTTCCTTTCTT-T	naA-E1	AB009724
	2	ATCATTTCCTGTCTTCC	naA-E2	AB009725
Larva-1	1	ATTATCTTATGTTTT-T	naC-F2	
	2	ATTATTTCCTTTCTT-T	naA-E1	
Larva-2	1	ATTATCTTATGTTTT-T	naC-F2	
	2	ATCATTTCCTGTCTTCC	naA-E2	
A. nasuta-A colony C (egg donor)	1	ATTATTTCCTTTCTT-T	naA-C1	AB009720
	2	ATTATCTCTATGCGC-T	naA-C2	AB009721
A. formosa-A colony M (sperm donor)	1	ATCATTTCCTGTCTT-C	foA-M1	AB009751
	2	ATTACTTCCTTTCTT-T	foA-M2	AB009752
Larva-1	1	ATTATTTCCTTTCTT-T	naA-C1	
	2	ATCATTTCCTGTCTT-C	foA-M1	
A. florida colony N (egg donor)	1	AT-ATTAGCTTTCTT-T	flo-N1	AB009771
	2	AT-A-TAGCTTTCTT-T	flo-N2	AB029484
A. nobilis colony O (sperm donor)	1	AT-TTTTGCTTTCT--T	nob-O1	AB009762
	2	AT-ATTTGCTTTCTT-T	nob-O2	AB009763
Larva-1	1	AT-A-TAGCTTTCTT-T	flo-N2	
	2	AT-TTTTGCTTTCT--T	nob-O1	

[a] Only the nucleotides at 17 positions revealing differences between the four parental alleles are shown. Nucleotide positions are 11, 25, 45, 52, 61, 114, 132, 133, 143, 151, 166, 167, 186, 223, 247, 248, and 251 in the full-length sequences. Hyphens represent gaps.

larvae in response to induction (data not shown). For technical reasons, we could not examine their further growth.

Gene Transmission to Hybrid Larvae

The presence of parental genomes in the hybrid larvae was confirmed by DNA analysis, using the mini-collagen gene as a marker. This is a single-copy gene encoded in the nuclear genome in acroporids (Wang et al. 1995). DNA was extracted from each individual colony, and the second intron region was amplified once by PCR, cloned, and sequenced. Two allelic sequences were determined for each colony by analyzing at least five clones. Similarly, two allelic sequences were determined for each individual larva and compared with the parental sequences.

Typical results are presented in table 2. Colony E of *A. nasuta*-A contained two allelic sequences, designated naA-E1 and naA-E2, while colony A of *A. nasuta*-C contained alleles naC-A1 and naC-A2. One hybrid larva produced in a cross between the two colonies contained two allelic sequences; one was identical to one of the egg donor alleles, naA-E1, and the other was identical to one of the sperm donor alleles, naC-A2. A second larva contained the allelic sequences naA-E2 and naC-A2. Another example is a cross between *A. nasuta*-A and *A. formosa*-A (fig. 2*A* and *E*). A hybrid larva contained two allelic sequences, of which one was identical to ''allele 1'' of one parent (naA-C1) and the other was identical to ''allele 1'' of the other parent (foA-M1). The allelic sequences separated and were transmitted in a Mendelian manner, as shown in table 2. Similar results were obtained for hybrid larvae produced in five other combinations examined (data not shown). These results show unambiguously that normal fertilization can occur between different *Acropora* species and that genetic information is transmitted to hybrid descendants with normal diploid inheritance patterns.

Molecular Phylogeny

The second intron region of the mini-collagen gene was sequenced for the individual colonies used for crossing experiments. Two heterozygous alleles were identified in the majority of DNA samples examined. In eight cases, however, only one allele was identified, suggesting either that the colony was homozygous or that the PCR failed to amplify the second allele due to a nucleotide substitution within the primer-binding site.

Fig. 3 shows a phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei 1987) for 49 alleles, including 1 allele of *A. donei* (fig. 2*I*), which was previously determined for a genomic clone (Wang

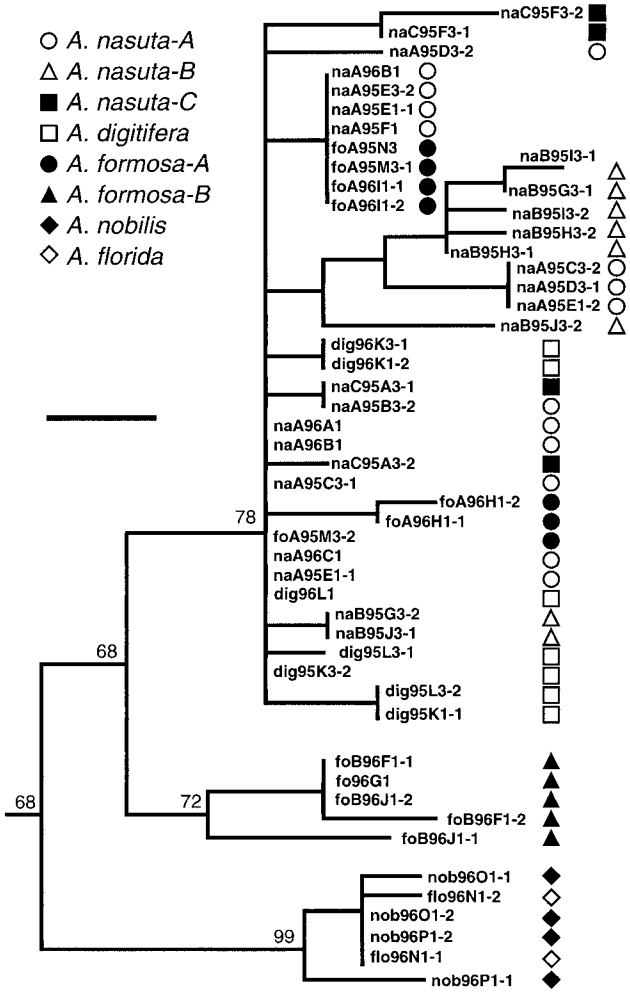

FIG. 3.—Molecular phylogeny. The DNA phylogenetic tree for the second intron region of the mini-collagen gene was constructed by the neighbor-joining method with compensation by Kimura's (1980) two-parameter method. Short branches (<0.5 nucleotide substitutions per gene) were removed after transforming per-site real values to per-sequence integer values (Saitou 1996). The root was determined by using an *Acropora donei* sequence. The scale bar represents 1% difference. Bootstrap values (% of 1,000 replicates) are shown above major branches. Colony numbers are represented by seven letters; ''-1'' or ''-2'' following a number indicates the specific allele for each colony. Absence of ''-1'' or ''-2'' after the colony number indicates that only one allele was identified for the colony. Species are indicated by symbols shown at upper left. Nucleotide sequences of all alleles are available in the DDBJ DNA database under accession numbers AB009715–AB009776 and AB029484.

et al. 1995), as the outgroup. The unweighted pair grouping method with arithmetic means (UPGMA) method gave similar results (data not shown). The alleles from five species (*A. nasuta*-A, *A. nasuta*-B, *A. nasuta*-C, *A. digitifera,* and *A. formosa*-A) formed a large cluster instead of forming separate clusters for each species. A close genetic relationship was also found between *A. florida* and *A. nobilis,* which cross-fertilized (table 1) in spite of morphological differences (fig. 2*G* and *H*). Alleles from *A. formosa*-B formed a cluster separate from all the other species. *Acropora formosa*-B did not cross-fertilize with any other species tested, not even with *A. formosa*-A or *A. nobilis,* which have very similar morphologies (fig. 2*E–G*). As expected, the *A. donei* allele used as the outgroup was very isolated from the alleles of all the other species. *Acropora donei* is reproductively isolated from the other species, since it spawns 2–3 h before the mass spawning.

Discussion

Cross-fertilization was observed in 8 out of the 44 combinations of 8 mass-spawning species of the genus *Acropora* tested to date. The frequency of interspecific fertilization was similar to that reported for the same genus in Australia (5 out of 38 combinations involving 12 species; Wallace and Willis 1994; Willis et al. 1997). The correlation of fertilization specificity and morphological types suggests that the hybrids cannot be regarded as morphological variants within a single species. In nature, a certain frequency of hybrids should be produced in mass spawnings, because eggs are exposed to high concentrations of sperm released from neighboring colonies of different species.

A striking finding of our study is that of cross-fertilization between species that have very different morphologies. We also revealed reciprocal interspecific fertilization in *Acropora* for the first time, shedding new light on the nature of hybrid formation in this genus. This also supports the hypothesis that fertilization truly occurs between distinct species.

We also revealed diploid hybridization in *Acropora.* All eight species that we examined possess 28 chromosomes (Kenyon 1997). Our DNA analyses confirmed the diploidy of the mini-collagen locus (fig. 3). In addition, we demonstrated that the two alleles were transmitted from the parents to the descendants in a Mendelian manner (table 2). This is the first molecular evidence that the offspring of coral produced in experimental crosses are really hybrids. Hybridization is thought to be fairly common among these diploid species.

Since hybrids do not necessarily express intermediate phenotypes, some *Acropora* hybrids might form unexpected morphologies and currently be recognized as independent species morphologically. Ungerer et al. (1998) suggested that rapid speciation in plants resulted from diploid hybridization. Similarly, hybridization might mediate an increase in species and morphological diversity in mass-spawning corals.

The degree of reproductive isolation in gamete-spawning animals may be largely due to egg-sperm recognition systems. Sperm proteins, lysin in abalone and bindin in sea urchin, play a major role in species-specific binding of sperm to eggs. Positive selection generates extensive amino acid substitutions in lysin and bindin and leads to species-specific egg-sperm recognition as a reproductive barrier between closely related species (Lee, Ota, and Vacquier 1995; Metz and Palumbi 1996). In *Acropora,* egg-sperm recognition systems might be responsible for the reproductive relationships between species, although there is no experimental evidence of this.

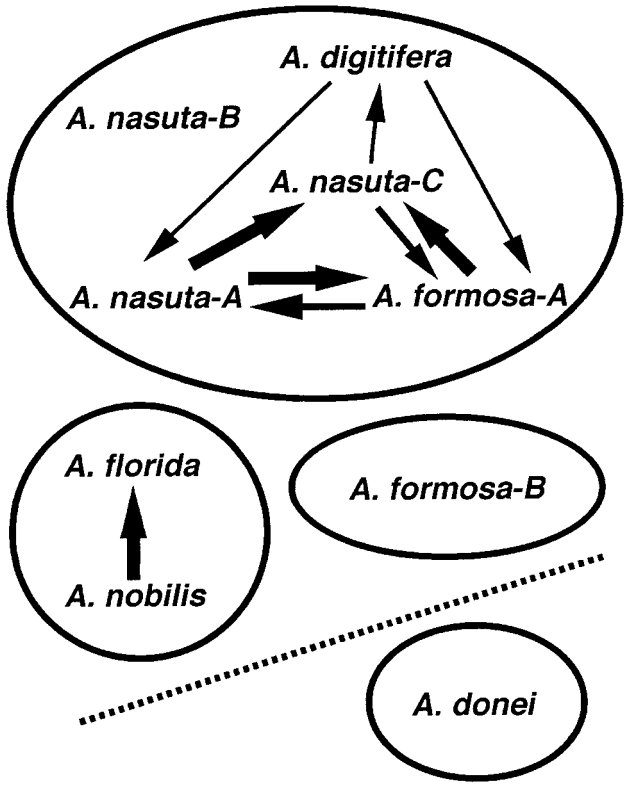

FIG. 4.—Schematic diagram of genetic and reproductive relationships among nine species in the genus *Acropora.* Circles indicate species very closely related to each other at the DNA level (fig. 1). Arrows indicate that sperm from species at the origin can fertilize eggs of species at the ends of the arrows. The thickness of an arrow indicates the frequency of cross-fertilization: thick indicates >50%, moderate indicates 20%–50%, and thin indicates 3%–20% of average values (table 1). The dotted line represents a reproductive barrier between species involved in mass spawning and those not involved in mass spawning.

Figure 4 schematically summarizes the reproductive and genetic relationships of the species. The circles enclose genetically related groups suggested by DNA phylogenetic analyses (fig. 3), and arrows indicate cross-fertilization (table 1). The eight mass-spawning species were divided into three genetic groups, and interspecific fertilization occurred only within groups. The most important findings are typified by the group of species enclosed in the largest circle in figure 4. This group contains five species, including some with very different morphologies, such as *A. nasuta*-A and *A. formosa*-A (fig. 2*A* and *E*). DNA phylogenetic analyses, however, suggest that these species are as closely related to each other as individuals within the same species. DNA markers will not detect natural hybrids, if they exist, since hybridization occurs between species with interconnected genetic relationships. *Acropora florida* and *A. nobilis* may form a second group of species. No genetic groups are morphologically similar. Species with very different morphologies, such as *A. nasuta* and *A. formosa-A* (fig. 2*A* and *E*) and *A. florida* and *A. nobilis* (fig. 2*G* and *H*), were closely related, while three dendritic species, *A. formosa*-A, *A. formosa*-B, and *A. nobilis*, were unrelated to each other even though their morphologies were very similar (fig. 2*E–G*).

In addition to the mini-collagen gene phylogeny, a preliminary study of ribosomal DNA revealed similar genetic relationships between two *Acropora* species (Odorico and Miller 1996) that cross-fertilized in vitro (Wallace and Willis 1994). This suggests that an interconnected DNA phylogeny is common to hybridizing species and independent of loci.

Altogether, the data suggest that *Acropora* contains groups of species that have imperfect reproductive isolation and genetic connectivity. These characteristics are consistent with the concept of a species complex under the processes of separation, or the fusion of multiple species, ''metaspecies'' or ''syngameon,'' assumed in the reticulate evolutionary hypothesis for mass-spawning corals (Veron 1995).

Two scenarios, not mutually exclusive, can be invoked to explain such species groups. The first is based on the process of speciation. The five species in the large circle in figure 4 are currently becoming more reproductively isolated from a single common ancestral species that was highly polymorphic morphologically. Each species has a fixed species-specific morphology. According to this scenario, for example, *A. nasuta*-B speciated from a common ancestor of *A. nasuta*-A and still remains morphologically similar and shares DNA polymorphism, although it has recently achieved reproductive isolation. However, it is difficult to conceive how several species arose from a single ancestral species sympatrically at the same time, and that very different morphologies, such as those seen in *A. nasuta* and *A. formosa,* have become fixed in each species without fixing DNA polymorphism.

The alternative scenario invokes interspecific gene introgression via the hybrids produced by mass spawning. On an evolutionary timescale, five once-isolated species have recently become able to cross-fertilize in the mass spawning due to changes in sea level or ocean currents. *Acropora nasuta*-B could be included in this group if there is an as yet unidentified species that can hybridize with both *A. nasuta*-B and another member of the group. If the hybrids are fertile and can backcross with their parental species, then the transmission of genes from one species to another serves as a channel for interspecific gene flow. Such channels may be very narrow initially, and widen with time with repeated hybridization and backcrossing such that the hybridizing species come to share a gene pool as a result of extensive gene introgression.

Irrespective of the scenario, the interconnection of the many *Acropora* species described in this study reveals characteristics consistent with a reticulate evolutionary history examined at a point in time. Mass spawning may have facilitated the unique evolutionary history of corals.

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