

NANCY KNOWLTON AND ANN E. BUDD

Introduction

Species are a fundamental unit of biodiversity because they represent distinct and independent lineages, often with characteristic ecological requirements, life histories, and physiologies. In some groups they are also morphologically well defined, so that recognizing species is relatively easy. Mayr (1963), for example, estimated that only 5% of birds belong to problematic complexes of sibling species. Although more recent analyses have increased this figure somewhat, it remains the case that a marine biologist or paleontologist with a pair of binoculars and a field guide can often correctly identify the hummingbirds of Panama. The corals of Panama are, however, a different story, as there is little agreement on species boundaries in many of the important genera, or their relationships to similar taxa elsewhere.

While the problematic nature of scleractinian coral species is widely recognized (Budd 1990; Veron 1995; Wallace and Willis 1994; Willis et al. 1997), there is no consensus as to why this problem exists. Veron (1995) has argued that species boundaries in corals may not be clear-cut because of extensive and complex patterns of hybridization. The relatively simple morphology of corals may make developmental catastrophes less likely in hybrids, and the fact that many different coral species spawn at the same time ("mass spawning") may give their gametes numerous opportunities for interspecific fertilizations (Veron 1995). Alternatively, species may be quite discrete in terms of their breeding biology, but exhibit much overlap in the characters traditionally used to tell them apart. Phenotypic plasticity (Willis 1985), morphological stasis (Potts et al. 1993; Budd, Johnson, and Potts 1994), slow rates of molecular evolution (Romano and Palumbi 1996, 1997; van Oppen et al. 1999), and relatively recent origins (Palumbi 1994; Budd, Stemmann, and Johnson 1994) combined with long generation times (Potts 1984) could all contribute to the difficulty of identifying reproductively discrete groups. In

any case, the problem of recognizing species gets even worse as one moves from sympatric morphotypes to allopatric populations (e.g., Johnson 1991) because there is no commonly accepted criterion for species status in allopatry (Cracraft 1989; Knowlton and Weigt 1997; Knowlton 2000).

Although enthusiasm for Veron's reticulate view is widespread, the data needed to test it are largely lacking. This is worrisome, because the plant literature is rife with supposed examples of ancient or ongoing hybridization that have turned out to be false or overstated upon closer investigation (Howard et al. 1997; Rieseberg 1997). The best support comes from the west Pacific. Several studies (Willis et al. 1997; Hatta et al. 1999) have shown that fertilization success is often high in the laboratory between conventionally defined species in the genera *Acropora*, *Montipora*, and *Platygyra*. In *Acropora*, patterns of chromosome numbers (Kenyon 1997), multiple, highly distinctive ITS sequences within nominal species (Odorico and Miller 1997), and phylogenetic analyses (Hatta et al. 1999) suggest a possible history of past hybridization. In *Platygyra*, distinct morphotypes show little or no evidence for either genetic differentiation or barriers to interbreeding (Miller 1994; Miller and Babcock 1997; Miller and Benzie 1997). On the other hand, despite the suggestive nature of the data for these corals, no genetic evidence for routine hybridization in the field exists (e.g., abundant F_1 s), the viability and fertility of F_2 s remain to be demonstrated, and shared ancestral polymorphisms are an alternative explanation for some of the genetic patterns. For Caribbean corals, there are almost no data of the type needed to examine this problem.

Montastraea annularis as a Model System

Montastraea annularis sensu lato provides an important example for exploring species boundaries in Caribbean corals. This coral is the dominant reef builder of the region (Goreau 1959) and has been so for millions of years (Budd, Stemann, and Johnson 1994). As such, it has been a model organism for a variety of topics, including phenotypic plasticity, coral bleaching, stable isotopes, and symbiosis (e.g., Graus and Macintyre 1976; Fairbanks and Dodge 1979; Dustan 1982; Porter et al. 1989; Szmant and Gassman 1990; Fitt et al. 1993; Dunbar and Cole 1993; Gleason and Wellington 1993; Rowan and Knowlton 1995; Rowan et al. 1997).

For decades, *M. annularis* sensu lato was considered the archetypal generalist coral (Connell 1978) with a distribution ranging from the intertidal to over 80 m (Goreau and Wells 1967). The extensive variability in colony morphology (heads, columns, and plates) exhibited over this

depth range was believed to be an adaptive response to differing light levels (Graus and Macintyre 1976), despite the apparent absence of intermediates (Graus 1977). More recently, however, a number of features have been found to covary with the different types of colony morphologies, including allozymes, aggressive behavior, ecology, growth rate, life history, corallite morphometrics, and stable isotopes (Tomascik 1990; Knowlton et al. 1992; Van Veghel and Bak 1993).

Weil and Knowlton (1994) consequently resurrected two previously synonymized species (*M. faveolata* and *M. franksi*) making, together with *M. annularis* sensu stricto, a total of three species in the complex. This decision was based on the widely accepted principle of concordance (Avice and Ball 1990), namely that a broad array of traits would not consistently covary if reproductive barriers between taxa were absent. Van Veghel and Bak (1993) also found comparable differences among the three morphotypes in Curaçao, whose reefs lie about 1,000 km from those of Panama, but argued that species-level designation was inappropriate without fixed, diagnostic differences. Szmant et al. (1997) also pointed out that many colonies in the northern Caribbean do not easily fit into the categories defined by Weil and Knowlton (1994), although no genetic data or morphological analyses are available to determine whether such colonies are likely to be hybrids or undescribed taxa.

Nonmorphological studies have often provided important early clues to the existence of unrecognized sibling or cryptic species (Lang 1984). Ultimately, however, morphological characters will be needed to recognize these taxa in the fossil record and to reconstruct their origins spatially and temporally. Below we review the nonmorphological evidence supporting the species described by Weil and Knowlton (1994), and then present results of our recent attempts to distinguish them morphologically.

Nonmorphological Approaches

Reproductive Biology

Differences in reproductive biology provide important characters in their own right, as well as possible clues to the underlying cause of genetic barriers between cryptic species. Such evidence falls into two broad classes: differences in the timing of reproduction, and fertilization or early developmental failures (which are separate in mechanism but often difficult to distinguish in practice).

Members of the *M. annularis* complex, like many other major reef builders (Harrison et al. 1984), spawn on just a few nights of the year. At many sites, spawning occurs 7–8 days after the full moon in August, or in September if the August full moon is very early (Gittings et al. 1992;

Knowlton et al. 1997; Szmant et al. 1997). *Montastraea* spawns 1 month earlier in Bermuda (Wyers et al. 1991) and 1 month later in Curaçao (Van Veghel 1994), perhaps due to local differences in the annual temperature cycle (Van Veghel 1994). Nevertheless, at a variety of sites (e.g., Panama, Curaçao, and Florida), the three taxa show considerable overlap in the dates of peak spawning.

Across the Caribbean, spawning generally occurs 1.5–4.5 hours after local sunset time. However, in Panama (Knowlton et al. 1997 and unpublished data), the Florida Keys (Szmant et al. 1997), and the Texas Flower Gardens (Hagman, Gittings, and Deslarzes 1998), there is a consistent 1–2-hour difference in the time of spawning between *M. franksi* and the other two taxa. Although Van Veghel (1994) reported simultaneous spawning by the three taxa in Curaçao, recent observations (Van Veghel and Knowlton, unpublished data) suggest that *M. franksi* spawns earlier than the other two taxa at this site as well. The time of spawning can be accelerated in all three taxa by providing an artificially early sunset in the laboratory (Knowlton et al. 1997 and unpublished data from Panama), a phenomenon also reported for some Pacific corals (Babcock 1984; Hunter 1989; Harrison 1989).

A difference of 1–2 hours in spawning time should dramatically reduce the potential for gene exchange in the field, despite long sperm life spans under laboratory conditions. For example, sperm of *M. franksi* could either move off the reef or become too dilute to be effective by the time eggs of the other species are released. In support of this, Oliver and Babcock (1992) found that fertilization of previously collected eggs exposed to water taken from over the reef was high immediately after conspecifics spawned in the field but dropped sharply for water collected from the same site 2 hours later. Dilution can also decrease the longevity of sperm (Levitan et al. 1991), so that laboratory estimates of longevity at high concentrations may not be relevant to naturally occurring situations.

Synchronous spawning, such as that exhibited by *M. annularis* and *M. faveolata*, does not guarantee cross-fertilization. The importance of chemical blocks to fusion between eggs and sperm of different species has been documented in both echinoderms (Metz et al. 1994) and mollusks (Swanson and Vacquier 1998), and may play a major role in the evolution of new species in many groups that spawn their gametes into the water column (Palumbi 1994). Chemicals that attract sperm to eggs also have the potential to diverge and promote speciation (Coll et al. 1994).

Preliminary studies of *Montastraea* fertilization under laboratory conditions in Panama (Knowlton et al. 1997; Levitan and Knowlton, unpublished data) suggest that *M. annularis* and *M. franksi* cross-fertilize readily under laboratory conditions, but that *M. faveolata* may not cross well with the other two taxa, particularly *M. annularis*. Thus the least

successful crosses are between the two taxa that spawn simultaneously. As with many broadcast spawners (Heyward and Babcock 1986), crosses using gamete bundles from the same colony (selfing) yielded few successful fertilizations (Knowlton et al. 1997; Levitan and Knowlton, unpublished data).

Reproductive barriers between *M. faveolata* and *M. franksi* were also found by Hagman, Gittings, and Vize (1998) on the Texas Flower Garden reefs; thus studies from at least two widely separated locations indicate that *M. faveolata* may be limited in its potential to cross-fertilize with the other two taxa. In contrast, Szmant et al. (1997) reported considerable cross-fertilization in all possible combinations (as well as failures to fertilize within taxa) in corals from the Florida Keys. It is difficult to know whether the differences among studies stem from actual biological differences between regions or differences in technique.

Symbiotic Associations

Like all reef-building corals, *Montastraea* hosts dinoflagellate symbionts (termed "zooxanthellae") of the genus *Symbiodinium*. The discovery that these and other corals can host different types of zooxanthellae (Rowan 1998) complicates interpretation of other differences among coral taxa, because the characteristics of the coral might be influenced by the type of symbiont being hosted. In the Caribbean, there are at least four major clades of zooxanthellae, and their distribution in *Montastraea* appears to be determined by ambient light levels and other less well understood factors, both across the reef and within individual colonies (Rowan and Knowlton 1995; Rowan et al. 1997; Toller et al. in press a, in press b). There are subtle differences among the coral taxa in the patterns of symbiont association, but the four taxa of zooxanthellae have broadly similar depth distributions in all three members of the *M. annularis* complex (Rowan and Knowlton 1995; Rowan et al. 1997; Toller et al. in press b). This suggests that the substantial differences in colony morphology exhibited by the three coral taxa at depths where they co-occur cannot be readily attributed to differences in their symbionts.

Aggressive Behavior

Lang (1971) was the first to document that a certain form of aggressive behavior, termed "extracoelenteric digestion," was associated with species boundaries. When colonies of different species are placed next to each other, the dominant species everts its stomach and digests away the adjacent portions of its neighbor within 24–48 hours (Lang 1973). In the case of *Montastraea*, the three taxa show a linear dominance hierarchy, with *M. franksi* dominant over both other taxa and *M. faveolata* dominant over *M. annularis* (Knowlton et al. 1992; Van Veghel and Bak

1993; Weil and Knowlton 1994). This reaction is highly consistent and provides a quick and dirty field assay for recognizing genetically distinct forms in sympatry.

Biochemical and Genetic Analyses

Genetic analyses have considerable potential to reveal cryptic species, particularly in sympatry, because such differences are difficult to explain in the absence of barriers to gene flow between taxa (Avice and Ball 1990; Avice 1994; Thorpe and Solé-Cava 1994). When several independent genetic markers are consistently associated with particular morphologies, the evidence for cryptic species is compelling.

Earlier work on nine polymorphic allozyme loci from Panama and Curaçao showed that *M. franksi* and *M. annularis* are more closely related to each other (Nei's D of 0.06–0.07) than either are to *M. faveolata* (Nei's D of 0.13–0.26); the latter is distinguished by a nearly fixed difference at one locus (Knowlton et al. 1992; Van Veghel and Bak 1993; Weil and Knowlton 1994). Cluster analysis including populations from both sites generates groupings consistent with morphologically based characterizations (e.g., *M. franksi* from Curaçao is more similar genetically to *M. franksi* from Panama than to other Curaçao taxa; Van Veghel, Weil, and Knowlton, unpublished data).

More recently, DNA-based analyses have been tried (Lopez and Knowlton 1997; Lopez et al. 1999; Medina et al. 1999), typically using gametes as a source of DNA because they are free of symbionts (Szmant 1991). Attempts to find diagnostic sequence differences by targeting specific genes (ITS-1 and ITS-2 of ribosomal DNA, a beta-tubulin intron, and the mtDNA COI gene) have been unsuccessful, although there appear to be minor frequency differences in a few nucleotide positions (Lopez and Knowlton 1997). The failure to find diagnostic differences may be related to very slow rates of molecular evolution. For example, Medina et al. (1999) found only 2.4% divergence in 658 base pairs of COI sequence between *Montastraea cavernosa* and the members of the *M. annularis* complex, with all but one of thirteen individuals of the latter being genetically identical. Given that *M. cavernosa* probably diverged from the lineage leading to *M. annularis* sensu lato approximately 50 million years ago (see below), these data imply an exceptionally slow rate of molecular evolution of less than 0.05% per million years. It is thus not surprising that the members of the *M. annularis* complex, which separated less than 10 million years ago (Ma; see below), show essentially no divergence.

The generally low level of variability in ITS, beta-tubulin, and COI sequences suggests that approaches that examine a broader portion of the genome might be more successful. Analyses based on amplified fragment length polymorphisms (AFLPs; Vos et al. 1995; Mueller et al. 1996) re-

vealed two markers able to distinguish *M. faveolata* from *M. franksi* and *M. annularis* (Lopez and Knowlton 1997; Lopez et al. 1999). For one of these, the DNA from diagnostic bands was sequenced and primers were designed for the region, allowing the insertions/deletions that distinguish *M. faveolata* from the other two taxa to be identified (Lopez et al. 1999). The differences revealed by analysis of gametes can also be seen when somatic tissues are analyzed. As with allozymes, there were no fixed or nearly fixed differences between *M. annularis* and *M. franksi*; however only twelve of many hundred possible primers have been screened.

Morphology and the Fossil Record

Analyses of Recent Material

Morphology is the traditional tool of systematics, and it is the only tool for identifying species in fossil deposits. Thus studies of the origination and extinction of species and their stability through geologic time require morphological characters. Nevertheless, use of morphology in scleractinian systematics has been hampered by (1) a general shortage of morphological characters, (2) a lack of discrete morphological characters (many consist of quantitative measurements or counts), and (3) high phenotypic plasticity, both documented (Foster 1979; Willis 1985; Bruno and Edmunds 1997) and inferred. Most of the classic monographs have distinguished species using a few imprecisely defined measurements or counts (e.g., in *Montastraea*; see Vaughan 1919; Veron et al. 1977), and many workers are unwilling to accept species that overlap morphologically, either in single characters or in character combinations (e.g., Best et al. 1984; Riegel and Piller 1995). This perspective dates back at least to Wood-Jones in the early 1900s, who stated that "from the study of the life of the colony in different surroundings, and from repair of injury, and death, in unsuitable habitats, I think it will be seen that the number of the true species of corals is by no means so great as is at present supposed . . . In very many cases one single colony could be found to provide several types of growth, that if presented as fragments, would be deemed to merit individual description of species" (Wood-Jones 1907, 554–55). The end result in some cases has been lumping of clearly distinct taxa (e.g., Zlatarski and Estalella 1982).

In the southern Caribbean, *Montastraea annularis*, *M. faveolata*, and *M. franksi* can be visually distinguished in the field using characters related to overall colony shape and the colony growing edge. However, initial morphological analyses of corallite measures revealed no single diagnostic difference among the three species in three key characters that have traditionally been used to distinguish species of *Montastraea*: number of septa per corallite, calice diameter, and calice spacing (Knowlton

et al. 1992; Weil and Knowlton 1994). Univariate analyses of variance and canonical discriminant analysis of traditional measurements and counts did show statistically significant differences among the species in both Panama (Weil and Knowlton 1994) and Curaçao (Van Veghel and Bak 1993). Nevertheless, because the distributions of these data overlap among species, specimens cannot be identified with complete confidence using these methods.

Inspired by the pioneering analyses of Cheetham (1986, 1987), we are beginning to develop statistical protocols that use new, more refined and biologically meaningful morphological characters that are evenly sampled across each colony. Our initial efforts have focused on capturing three-dimensional landmark data on corallite surfaces using a Reflex microscope, and exploring a variety of different geometric approaches to analyze the data (Budd, Johnson, and Potts 1994; Budd and Johnson 1996; Johnson and Budd 1996). In these analyses, three-dimensional Cartesian coordinates have been obtained for 34 landmarks (fig. 4.1A) on calices of 21 colonies (5 *M. annularis*, 7 *M. faveolata*, and 9 *M. franksi*). Six calices were digitized on samples from the top, middle, and edge of each colony. Size and shape coordinates (Bookstein 1991) were calculated for selected triplets of landmarks using a program for three-dimensional landmark analysis written by K. G. Johnson (Budd, Johnson, and Potts 1994). Centroid size was used to estimate calice size and spacing; shape coordinates were determined by studying triangles formed by selected triplets of landmarks (see Budd, Johnson, and Potts 1994 for details).

The size and shape coordinate data were then analyzed using two multivariate statistical procedures, cluster analysis and canonical discriminant analysis, and the digitized colonies were grouped into species by splitting to the highest levels of statistical significance. This approach follows Jackson and Cheetham (1990, 1994), who used breeding experiments and protein electrophoresis to confirm that morphospecies of bryozoans defined by splitting to the highest significance levels are genetically distinct. To select the size and shape coordinates that were included in cluster analyses, we explored the data distributions for outliers and correlations among characters. The size and shape coordinate data were then used to calculate Mahalanobis distances (Klecka 1980; Marcus 1993a) between samples, and the resulting distance matrix was analyzed using average linkage cluster analysis.

The resulting cluster dendrogram (fig. 4.2A) clearly shows three distinct groups that generally match independent field identifications of the three species based on overall colony shape. Canonical discriminant analysis indicates that the most important variables in discriminating species consist of characters related to the elevation and thickness of the septa

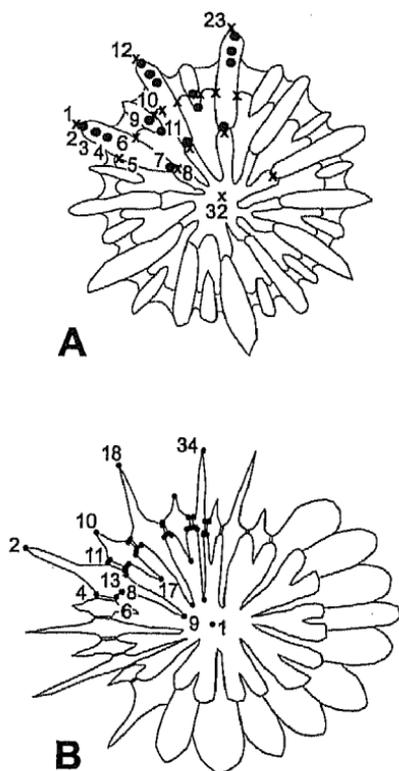


Fig. 4.1. Schematic diagrams showing the locations of landmarks whose Cartesian coordinates have been digitized. The landmarks consist of spatially homologous points selected to capture information about the structure and relief of the wall, septa, and columella (see Budd and Johnson 1996 for further details). *A*, 14 of 34 landmarks digitized on calices in three dimensions using a Reflex microscope. Centroid size was calculated by summing the squared differences between landmarks 1, 32, and 33 to a common centroid, and between landmarks 32, 33, and 34 to a common centroid. Shape coordinates were calculated for seven triplets of landmarks: primary septum elevation, 1-3-32; calical platform shape, 1-7-32; primary septum length, 1-8-32; costa height, 1-2-32; tertiary septum development, 9-10-11; tertiary costa development, 3-11-14; wall development, 4-5-6. *B*, 12 of 45 landmarks digitized on corallites in two dimensions using thin sections. Size and shape coordinates were calculated for 12 selected landmarks (1, 2, 4, 6, 8, 9-11, 13, 17, 18, 34) with landmarks 2 and 9 designated as the baseline.

and the shape of the septal margin (fig. 4.3A-C). The groups corresponding with *M. annularis* and *M. franksi* are the most similar, as was found in the genetic analyses described above. Interestingly, corallites from *M. franksi* consistently cluster with corallites from the sides of *M. annularis* columns. This pattern may reflect the fact that these corallites have similar, slow growth rates: *M. franksi* grows slowly throughout the colony,

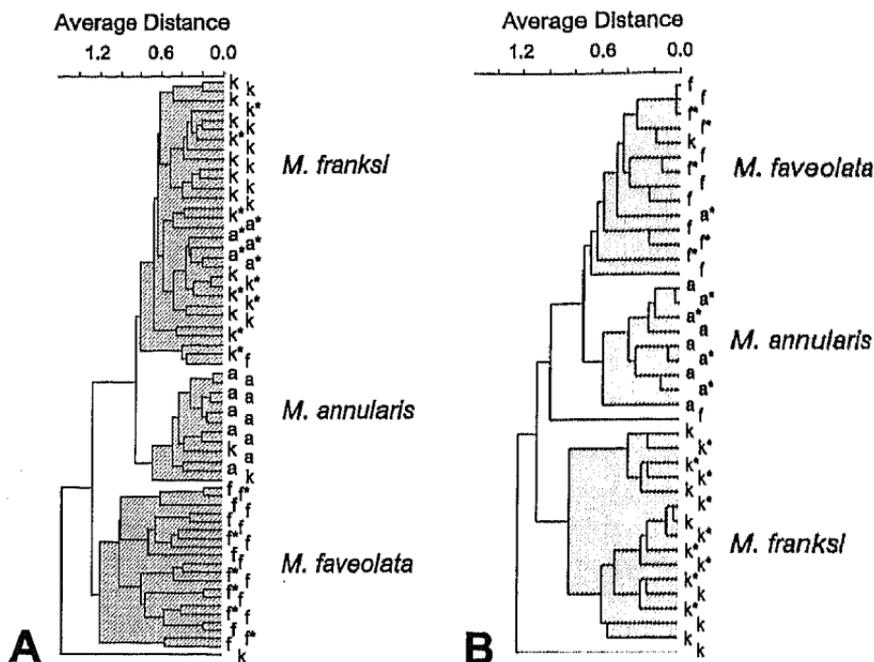


Fig. 4.2. Average linkage cluster dendrograms resulting from analyses of Bookstein size and shape coordinates. Each branch of the two dendrograms represents one sample consisting of six corallites on the top, middle, or edge of 21 colonies identified using field observations (5 colonies of *M. annularis*, 7 colonies of *M. faveolata*, and 9 colonies of *M. franksi*). Asterisks indicate samples of colony edges; a, k, and f refer to field identifications made respectively for *M. annularis*, *M. franksi*, and *M. faveolata*. A, Results based on three-dimensional analyses of the 14 landmarks shown in figure 4.1A (after Budd and Johnson 1996). B, Results based on two-dimensional analyses of the 12 landmarks shown in figure 4.1B.

while the sides of colonies of *M. annularis* columns are nearly senescent. This would suggest that, in these genetically similar corals, corallite morphology may converge when growth rates are similar.

Applying morphometric analyses of three-dimensional landmark data is difficult in fossil material, because many key diagnostic features in three dimensions are worn or recrystallized. Therefore, we conducted a pilot study in which 45 landmarks (fig. 4.1B) were digitized in two dimensions on thin sections of six corallites from the tops and edges of the same 21 colonies of the *M. annularis* complex as above. Size and shape coordinates were calculated for 11 selected landmarks (fig. 4.1B) using the Uni-graph 3 computer program (Marcus 1993b). As in the three-dimensional analyses, Mahalanobis distances were calculated using centroid size and nine pairs of shape coordinates, and entered into an average linkage cluster analysis. Like the three-dimensional analyses, three clearly distinct groups were detected (fig. 4.2B); however, *M. faveolata* and *M. annularis*