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Shallow mtDNA Coalescence in Atlantic Pygmy Angelfishes (Genus *Centropyge*) Indicates a Recent Invasion from the Indian Ocean

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Abstract

Pygmy angelfishes (genus *Centropyge*) are widespread and species-rich in the Indo-Pacific, but only three species are recognized in the Atlantic: *Centropyge resplendens* on the Mid-Atlantic Ridge, *Centropyge argi* in the Caribbean, and *Centropyge aurantonotus* in Brazil and the southern Caribbean. Atlantic species are distinguished only by color patterns and are very similar to *Centropyge acanthops* (*Cac*) in the western Indian Ocean, raising the possibility that pygmy angelfish recently invaded the Atlantic Ocean via southern Africa. To test this zoogeographic hypothesis, we compared a 454-bp segment of the mitochondrial DNA (mtDNA) control region among pygmy angelfishes of the subgenus *Xiphypops*, which includes the three Atlantic species, the Indian Ocean species, and an Indo-Pacific species [*Centropyge fisheri* (*Cfi*)]. The Indian Ocean species *Cac* is closest to the Atlantic species ($d = 0.059$) relative to *Cfi* ($d = 0.077$). The mtDNA genealogy indicates a colonization pathway from the Indian Ocean directly to the West Atlantic, followed by at least two waves of dispersal to the Mid-Atlantic Ridge. The gene tree for the three Atlantic species is polyphyletic, raising questions about taxonomic assignments based on color pattern. Mismatch distributions place Atlantic founder events and population expansions at about 250,000–500,000 years ago. Estimates of effective female population sizes from mismatch and coalescence analyses are consistent with founder events by tens of individuals in the western Atlantic, followed by expansions to several million individuals.

Reef fish faunas in the tropical Atlantic are less diverse than those in the Indo-Pacific but include most of the same taxonomic families and genera (Briggs 1974). These taxonomic groupings indicate evolutionary connections between Atlantic and Indo-Pacific reef faunas, but the age and tempo of these connections are largely unknown. The most recent direct connection between the tropical Atlantic and Indo-Pacific ended with the rise of the Isthmus of Panama, about 3.1–3.5 Ma (Coates and Obando 1996). An older connection between tropical Atlantic and Indian oceans closed with the collision of Africa and Eurasia, about 15–20 Ma (Hallam 1994). Despite these strong geological barriers, tropical faunas of the Atlantic and Indo-Pacific share more recent connections, indicating dispersal around southern Africa (Baldwin et al. 1998; Bowen et al. 1994, 1998, 2001; Dutton et al. 1999; Graves 1998; Smith and Heemstra 1986).

Two fast-moving currents flow around southern Africa. The tropical Agulhas Current moves westward and retroflexes to the southeast at the Cape of Good Hope (van Ballegooyen et al. 1991). The cold Benguela Current moves northward along the Atlantic coast but is deflected offshore by the warm southerly Angola Current. The role of these currents in promoting dispersal of tropical organisms has been largely unexplored although they have been instrumental in transporting southern African kelps and associated invertebrates into the South Atlantic (Edwards 1990). The geographic discontinuity in tropical habitat around southern Africa indicates that the cold water of the Benguela Current is a formidable barrier to dispersal into the Atlantic (Gibbons and Thibault-Botha 2002; Gibbons et al. 1995). Yet, the close relationship between many tropical organisms in the Atlantic and Indian oceans (Bowen et al. 1998, 2001; Rocha et al. 2005a) indicates recent (or late Pliestocene) dispersal.

Pygmy angelfishes (genus *Centropyge*; family Pomacanthidae) may be one example of a recent invasion into the tropical Atlantic. These species inhabit coral rubble at depths of

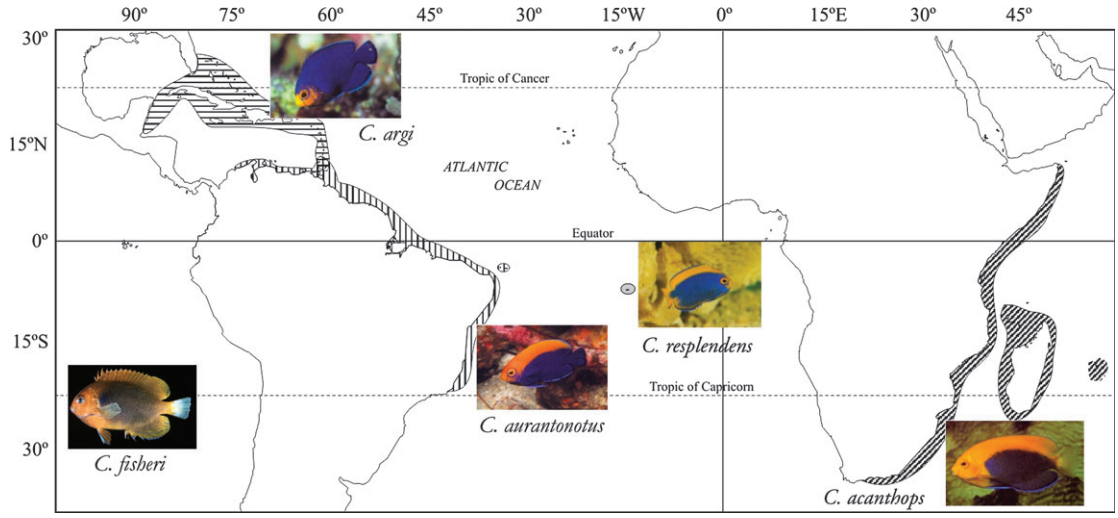

Figure 1. Map of the Atlantic and Indian oceans, with the range of each *Centropyge* species indicated by slanted lines (*C. acanthops* in the western Indian Ocean), gray shading (*C. resplendens* at Ascension Island on the Mid-Atlantic Ridge), vertical lines (*C. aurantonotus* in Brazil and the southern Caribbean, and horizontal lines (*C. argi* in the Caribbean). The Indian-Pacific outgroup, *C. fisheri*, is pictured at lower left (not representing true distribution). There is uncertainty about the distribution of *C. aurantonotus* along the northeastern coast of South America. Photo credit: J. E. Randall and G. R. Allen.

10–50+ m and have a pelagic larval phase of 30–35 days (Thresher and Brothers 1985). At least 34 species are distributed across all tropical seas except the East Atlantic and East Pacific (Allen 1985; Pyle 2003; Robertson and Allen 2002; Steene 1978). Only three species occur in the Atlantic, invoking the possibility that these species represent a recent radiation from the Indo-Pacific (see Briggs 1999a,b, 2003; Vermeij and Rosenberg 1993).

Centropyge argi Woods and Kanazawa 1951 (*Car*) (Cherubfish) occurs in the Caribbean, *Centropyge aurantonotus* Burgess 1974 (*Cau*) (flameback pygmy angelfish) occurs in Brazil and the southern Caribbean, and *Centropyge resplendens* Lubbock and Sankey 1975 (*Cre*) (resplendent pygmy angelfish) inhabits Ascension Island on the Mid-Atlantic Ridge (Figure 1; Allen 1985; Lubbock 1980; Randall 1968). The external appearance of these species is similar, supporting a hypothesis of monophyly. An Indian Ocean species, *Centropyge acanthops* Norman 1922 (*Cac*) (African flameback pygmy angelfish), occurs in the tropical waters of eastern Africa from Kenya to Natal, South Africa. The geographic proximity of this species to the Atlantic and its color pattern indicate a close relationship to Atlantic *Centropyge* species (Figure 1). In the only modern revision of pygmy angelfishes, Pyle (2003) placed these four species and the Indo-Pacific *Centropyge fisheri* Snyder 1904 (*Cfi*) in a monophyletic subgenus (*Xiphypops*) within *Centropyge*.

In this study, we use rapidly evolving mitochondrial DNA (mtDNA) control region sequences to evaluate several hypotheses about the origins and taxonomy of Atlantic *Centropyge*. We first determine whether the differences in coloration among putative Atlantic species are matched by molecular evolutionary partitions. Although mtDNA sequence data alone cannot validate species status, genetic divergences would support species designations for these color morphs (see Rocha 2004). The mtDNA control region has proved useful in resolving evolutionary partitions among color morphs of butterflyfishes (family Chaetodontidae; McMillan et al. 1999), a group closely affiliated with marine angelfishes (family Pomacanthidae).

Second, Bellwood et al. (2004) hypothesized that the Atlantic *Centropyge* species are the product of a recent colonization event from the Indian Ocean. The alternative is that Atlantic species are derived from the Indo-Pacific by dispersal through the eastern Pacific, predating the closure of the Panama Seaway. To test these alternatives, an Indo-Pacific member of the subgenus *Xiphypops*, *Cfi*, is included in our analysis of Atlantic and Indian ocean species.

A third issue is the timing of the proposed Atlantic colonization. Indian Ocean ancestors could have moved into the Atlantic during one of the mid-Pleistocene upwelling hiatuses off southern Africa, associated with ice age terminations (Chang et al. 1999; Peeters et al. 2004; Rocha et al. 2005a). More recent dispersal may also be possible. Warm-core gyres frequently bud off the Agulhas Current Retroflection and become entrained in the northward-moving Benguela Current (Flores et al. 1999; Penven et al. 2001). These fast-moving gyres are long lived and may facilitate the transport of warm-water species into the Atlantic. Although mtDNA control region comparisons cannot provide precise dispersal dates, they can distinguish between recent dispersals versus early Pleistocene or Pliocene events.

Finally, colonization pathways within the Atlantic Ocean are uncertain. The lack of *Centropyge* species in the eastern Atlantic may be due to the dispersal barrier created by the confluence of the southward-flowing Angola Current and the cold Benguela Current. Both these current systems would impede the northward dispersal of planktonic larvae along

the Atlantic coast of Africa, and the thermal regime of the Benguela Current may be a death sentence for tropical organisms. If larvae in warm-water gyres could breach the Benguela Current barrier, the subequatorial currents would facilitate dispersal into the central and western Atlantic. Under this scenario, the Mid-Atlantic Ridge islands of Ascension and St. Helena are obvious candidates for stepping-stone dispersals. However, the distributions of some marine invertebrates indicate dispersals from the Indian Ocean directly to the western Atlantic (Vermeij and Rosenberg 1993). Our phylogeographic analysis is consistent with this route from the Indian Ocean to the western Atlantic, followed by colonization of the Mid-Atlantic Ridge.

Materials and Methods

The study is based on *Car* from the Caribbean ($n = 14$), *Cau* from Brazil ($n = 17$), *Cre* from Ascension Island ($n = 15$), and *Cac* from Kenya ($n = 6$). Samples of *Cre* were collected at Ascension Island in 1997. Eight individuals of *Cau* were collected in Brazil and nine individuals were obtained from Brazil through the aquarium trade in 1997–1999. A Hawaiian specimen of *Cfi* was also obtained through the aquarium trade. [The widespread *C. flavicauda* has been synonomized with *Cfi* (Pyle 2003), so the latter is no longer a Hawaiian endemic.] The origins of specimens from commercial sources were verified by interviews with distributors and by inspection of importation documents from the original (reef location) supplier. Pygmy angelfishes are valuable aquarium trade species but have not yet been commercially bred in captivity. Thus, cultured fish do not introduce geographic errors into this study.

Tissue samples (muscle, gill, or both) collected from Ascension Island and Brazil were stored in a saturated salt-dimethyl sulfoxide buffer (Amos and Hoelzel 1991). Fish collected from the aquarium trade were euthanized, frozen, and dissected. Total genomic DNA was isolated from tissues with a lithium chloride procedure. A 454-bp segment of the mtDNA control region was amplified with heavy (5′-TTCCACCTCTAACTCCCAAAGCTAG-3′) and light (5′-AGCCTGGAAAGAAGCCCCGGCATGG-3′) strand primers (Lee et al. 1995). Polymerase chain reaction amplifications included an initial denaturing step at 94°C for 80 s, 35 cycles of amplification (42 s 94°C, 30 s 49°C, 55 s 72°C), and a final extension at 72°C for 150 s.

Single-stranded DNA sequencing reactions were conducted with fluorescently labeled dideoxy terminators, according to the manufacturer's recommendations (Applied Biosystems Inc., Foster City, CA). Labeled extension products were separated with gel electrophoresis and detected with an automated DNA sequencer (Applied Biosystems model 373A and 377) at the DNA Sequencing Core at the University of Florida. Sequences were aligned and edited with SEQUENCHER 3.0 (Gene Codes Corporation, Ann Arbor, MI). All specimens were sequenced in the forward direction, and select individuals from all species were sequenced in the reverse direction to ensure the accuracy of nucleotide assignments. Sequences are available in GenBank (accession nos. DQ343507–DQ343560).

Unbiased estimates of haplotype diversity (h) using equation 8.5 in Nei (1987) were produced with ARLEQUIN 2.0 (Schneider et al. 2000). We estimated the diversity index, $\theta = 2N_f\mu$, in two ways. The nucleotide diversity (π) was estimated by

$$\pi = \Sigma p_i p_j d_{ij},$$

where the p's are the frequencies of haplotypes and d is the sequence divergence between them (Nei 1987). When d is the number of mismatches between the sequences, θ_π is the average number of nucleotide mismatches in a mismatch distribution. The diversity index, θ_S, is estimated using the number of polymorphic nucleotide sites (Watterson 1975). Comparison of these two indices forms the basis for testing mtDNA sequences for fit to neutrality with Fu's F_S (Fu 1997). Significant negative values of F_S indicate an excess of low-frequency mutations arising from selection or from rapid population growth. Haplotype variability within and among the putative species of *Centropyge* was partitioned in an analysis of molecular variance and reported as Φ_{ST} values, using ARLEQUIN. Hierarchical log likelihood ratio tests of nested substitution models (MODELTEST 2.0; Posada and Crandall 1998) identified the Hasegawa-Kishino-Yano nucleotide substitution model (HKY+I+G) (Hasegawa et al. 1985) as the best fit to the sequences. With the Akaike information criterion (Akaike 1974), the general time reversal model (GTR+I+G) (Rodriguez et al. 1990) provided the best fit. The proportion of invariant sites (I) was 0.5553, and the gamma shape parameter (G) was $\alpha = 0.8357$, corresponding to a transition/transversion ratio of 10.16. Four substitution rate categories were used with the GTR model. These criteria were used to produce maximum likelihood (ML) trees with PAUP* 4.0 (Swofford 1998). Statistical parsimony (TCS 1.3; Clement et al. 2000) was used to construct a network of relationships among haplotypes. The HKY distance (Hasegawa et al. 1985) and the neighbor-joining (NJ) algorithm were used to produce 1,000 bootstrap trees (PAUP*). Bootstrap values were superimposed on a single tree produced with a complete set of data. Branches with bootstrap values greater than 50% were considered significant.

Alternative tree topologies were compared with the observed topologies of the ML trees using the procedures of Shimodaira (2002) (SH test) and Kishino and Hasegawa (1989) (KH test), as implemented in PAUP*, to test for evolutionary polarities in the ML trees. Likelihoods for each topology were fully optimized with 1,000 bootstrap replicates.

Time depths for phylogenetic separations in *Centropyge* are based on a sequence divergence estimate of 10%/million years (MY) between lineages or 5×10^{-8} within lineages (see Discussion). We used the shape of the nucleotide mismatch distribution to test for a model of exponential population growth (Rogers and Harpending 1992). Resampled distributions of Harpending's (1994) raggedness statistic were used to assess the fit of the mismatch distribution to the growth model. The $\theta = 2N_f u$ value was estimated from the distribution at the time before growth began and for

Table 1. Species, abbreviation, sample size, number of haplotypes, haplotype and nucleotide diversities, number of mutations per site, Fu's test of neutrality, F_S, and its probability

Species	Abbreviation	*N*	Number of haplotypes	*h* (SD)	Θ_π (SD)	Θ_s (SD)	F_s	*P*
Centropyge. argi	*Car*	14	14	1.00 (0.027)	0.0199 (0.0109)	0.0257 (0.0102)	−7.28	.002
C. aurantonotus	*Cau*	17	17	1.00 (0.020)	0.0221 (0.0119)	0.0255 (0.0097)	−9.90	.004
C. resplendens	*Cre*	15	14	0.991 (0.028)	0.0160 (0.0090)	0.0204 (0.0081)	−6.94	.004
Atlantic total		46	43	0.997 (0.005)	0.0226 (0.0117)	0.0347 (0.0104)	−24.69	<.001
C. acanthops	*Cac*	6	6	1.00 (0.096)	0.0332 (0.0200)	0.0347 (0.0170)	−0.40	.245

present-day populations (Rogers and Harpending 1992), where N_f is the effective female population size and u is the per-haplotype mutation rate (2.27×10^{-5}, based on the rate of 5×10^{-8}/site multiplied by 454 bp). The $\tau = 2ut$ value was estimated from the crest of the mismatch distribution and provides an estimate in generations (t) of the amount of time since population growth began. Generation times for *Centropyge* species were estimated to be 1 year, based on captive observation of maturity at 220 days and a lifespan of at least 3 years in Pacific species (Baensch F and Lutnesky MMF, personal communication).

Estimates of present-day female effective population size and growth were calculated with the coalescent approach (FLUCTUATE 1.0; Kuhner et al. 1995; 1998). FLUCTUATE develops ML estimates of the diversity index θ and the population growth parameter g with Metropolis-Hasting Markov chain-Monte Carlo simulations. We used 10 short Markov chains of 10,000 repetitions and two long chains of 200,000 repetitions. Present-day population sizes, $\theta_t = N_f 2\mu$, can be estimated by the relationship between growth and mutational time since coalescence, t, in the relationship

$$\theta_t = \theta_0^{-gt}.$$

The growth variable, g, is in units of $1/\mu$, and t is the average number of generations it takes one site to accumulate one mutation. Effective population sizes were estimated from $\theta_t = 2N_f\mu$, where μ is the per-site mutation rate within lineages (5×10^{-8}).

Results

We resolved 454 bp of the mtDNA control region in 53 pygmy angelfishes, including *Car* (Caribbean), *Cau* (Brazil and southern Caribbean), *Cre* (Mid-Atlantic Ridge), *Cac* (western Indian Ocean), and *Cfi* (Hawaii). Within the Atlantic, we observed 43 haplotypes among 46 sequences, with two haplotypes shared between *Cau* and *Cre* and one haplotype shared between specimens of *Cre*. Haplotype diversities were high ($h = 0.991$–1.0; Table 1). These values can be attributed in part to a high mutation rate in the mtDNA control region of reef fishes (McMillan and Palumbi 1997). Estimates of nucleotide diversity, θ_π, were lowest on the Mid-Atlantic Ridge (0.016 in *Cre*) and highest in the western Indian Ocean (0.033 in *Cac*; Table 1). Likewise, estimates of θ_S ranged from 0.020 in *Cre* to 0.035 in *Cac*. The larger values of θ_S relative to θ_π yielded negative values of F_S, which were significantly lower than 0.0 ($P < .001$) in all samples except *Cac*. These negative values indicate an excess of low-frequency haplotypes.

No population structure was detected between *Car* (Caribbean) and *Cau* (Brazil) in an analysis of molecular variance (AMOVA) ($\Phi_{ST} = 0.000$). However, significant structure was detected between *Cre* (mid-Atlantic) and both the Brazilian *Cau* ($\Phi_{ST} = 0.212$, $P < .001$) and the Caribbean *Car* ($\Phi_{ST} = 0.320$, $P < .001$). Divergences between haplotypes within the three Atlantic species as a whole are relatively shallow, ranging from $d_{HKY} = 0.00$–0.049 with means of 0.020, 0.021, and 0.016 for *Car*, *Cau*, and *Cre*, respectively.

The HKY and GTR ML trees differed only in the topology of a branch including five haplotypes of *Cre* and two haplotypes of *Cau* (Figure 2). Three clusters of haplotypes appear in the undirected ML trees. One cluster includes all the haplotypes from *Cac* and *Cfi* and a few haplotypes from *Cau* and *Car*. A second cluster contained haplotypes from *Cau* and *Car*, and a third cluster contained haplotypes predominantly from *Cre*, as well as a few haplotypes from *Cau*. Rooting by *Cac* places haplotypes in the western Atlantic (*Cau* and *Car*) at the base of the Atlantic Ocean tree.

A comparison of the topology of the five phylogroups (Figure 2a,b) with a topology in which *Cac* was placed as the outgroup was not significant with the KH test. This reflects the near equal genetic distance from *Cfi* and *Cac* to the Atlantic Ocean phylogroups. A set of simultaneous comparisons was also made between the phylogroup topologies shown in Figure 2 and six hypothetical topologies to evaluate which of the phylogroups were most derived. With the SH test, all the alternative topologies were rejected ($P < .05$), indicating that the likelihoods of the alternative topologies were not fully maximized relative to the trees shown in Figure 2. This indicates that the Atlantic phylogroups occupied the most derived position in the ML trees.

The NJ tree (Figure 3) illustrates the divergence between haplotypes and species. The Indian-Pacific species, *Cfi*, polarizes the tree and is the most distantly related to Atlantic species. Sequence divergences between *Cfi* and the remaining haplotypes averaged $d = 0.077$ (range 0.063–0.095). *Cfi* was set off from all the other haplotypes by bootstrap values exceeding 60%. The next deepest lineage included only haplotypes of *Cac* (Indian Ocean). Sequence divergence between haplotypes in *Cac* and the three Atlantic species averaged $d = 0.059$ (range 0.046–0.081). The cluster topology of Atlantic Ocean haplotypes was the same as that observed in the ML trees and depicted a polyphyletic relationship among

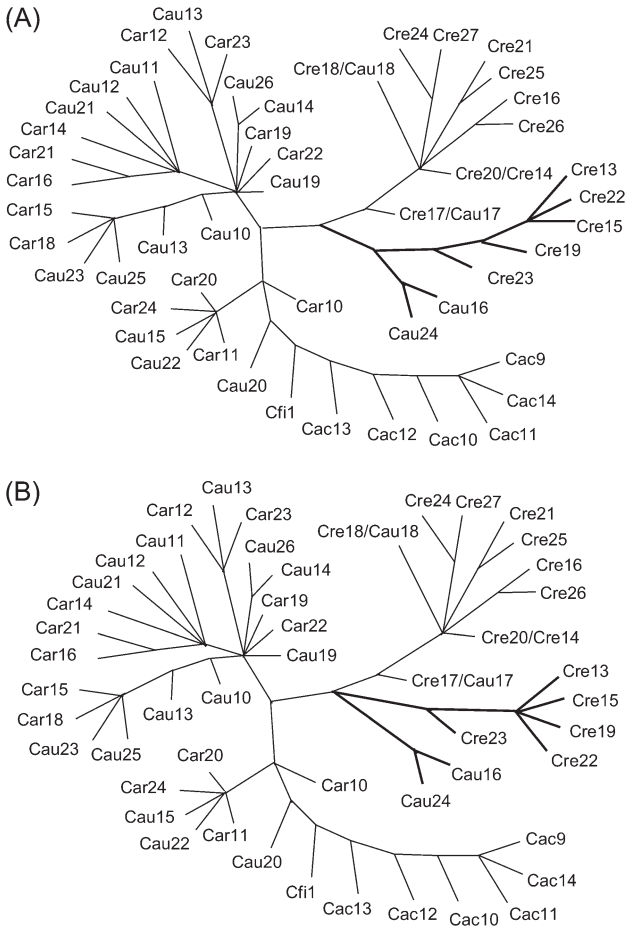

Figure 2. Maximum likelihood trees. (**A**) Tree based on the HKY-I-G substitution model, as chosen by the hierarchal test of log likelihood scores. (**B**) Tree based on the GTR-I-G substitution model, as chosen with the Akaike information criterion. Bold branches indicate differences between the topologies. Branch lengths are not scaled to divergences so that the distances between Atlantic, Indian (*Cac*), and Pacific (*Cfi*) species are not shown. Abbreviations of species names are defined in Table 1.

haplotypes in *Cau* and *Cre* and those in *Cau* and *Car*. Two clusters, including both *Cau* and *Cre* and one cluster including *Cau* and *Car*, had bootstrap support >50%.

A statistical parsimony network of haplotypes includes only Atlantic Ocean species because the haplotypes of *Cac* fell outside the 95% confidence interval (Figure 4). A large number of predicted (but unobserved) haplotypes characterize the haplotype network, a pattern likely due to the high mutation rate in the control region. This network corroborates the topologies of the ML and NJ trees in which haplotypes in *Cau* (Brazil-Caribbean) are closely related to haplotypes in both *Car* (Caribbean) and *Cre* (mid-Atlantic) but in which haplotypes of *Car* (Caribbean) and *Cre* (mid-Atlantic) do not occur in the same clusters. Haplotypes in *Cau* and *Car* form a large mutational network with branches that are incongruent with taxonomic designations. Two clusters of haplotypes, each including only *Cau* and *Cre*, are separated by seven mutations from the large *Cau-Car* complex.

The mismatch distributions of all species individually and the pooled sample of the three Atlantic species fit the exponential growth model of Harpending et al. (1993) and, hence, could be used to estimate historical and current demographic parameters. Mean mismatches ranged from 7.3 to 15.1 among species (Table 2). Estimates of the time since population expansion began in the Atlantic dated to about 250,000 years. The Indian Ocean species, *Cac*, however, produced a much larger estimate of about 400,000 years since population expansion. Estimates of the initial female (effective) population sizes in *Cau* and *Cac* were zero (founder events), and estimates of the initial population sizes in *Car* and *Cre* were only a few tens of females. Present-day effective population sizes were largest for *Cau* in Brazil and the Caribbean, numbering about 12 million females, and smallest for *Cre* in the mid-Atlantic, at 9,700 females. These values are consistent with a much lower quantity of habitat on the Mid-Atlantic Ridge island, relative to that in Brazil and the Caribbean.

The coalescence analysis does not depend on fit to a model of population expansion. Nonetheless, all the samples yielded large positive values of *g*, which are consistent with histories of population expansion (Table 3). Estimates of present-day female population sizes were larger than those produced from the mismatch analysis, but relative population sizes among species were similar to estimates from the mismatch analysis. Estimates of the effective female population sizes for *Car* and *Cau* in the western Atlantic were about 2 million and 12 million females, respectively. The corresponding value for *Cre* was about 10,000 females.

Discussion

The mtDNA survey of Atlantic pygmy angelfishes, and their relatives belonging to the subgenus *Xiphypops*, indicates a late Pleistocene dispersal from the Indian Ocean to the western Atlantic (on the order of a quarter to half million years), followed by more recent colonization of the Mid-Atlantic Ridge. This conclusion is consistent with the colonization pathway proposed by Bellwood et al. (2004). These results are also consistent with the paleontological findings of Vermeij and Rosenberg (1993), indicating that colonization can proceed from the Indian Ocean directly to the western Atlantic. Molecular data are useful in evaluating these previous hypotheses and in extending the conclusions of our illustrious predecessors. However, before dissecting these results, it is appropriate to address three caveats.

1. The molecular clock for the control region is uncertain and seems to vary among major taxonomic groups. Estimates of the corresponding mutation rate per site for bony fishes include 2.2–4.5%/MY between lineages for East African cichlids (Cichlidae; Sato et al. 2003), about 3%/MY for Australian rainbow fishes (Melanotaeniidae; Zhu et al. 1994), 3.6%/MY for snooks (Centropomidae; Donaldson and Wilson 1999), 5.6%/MY for Lake Victoria

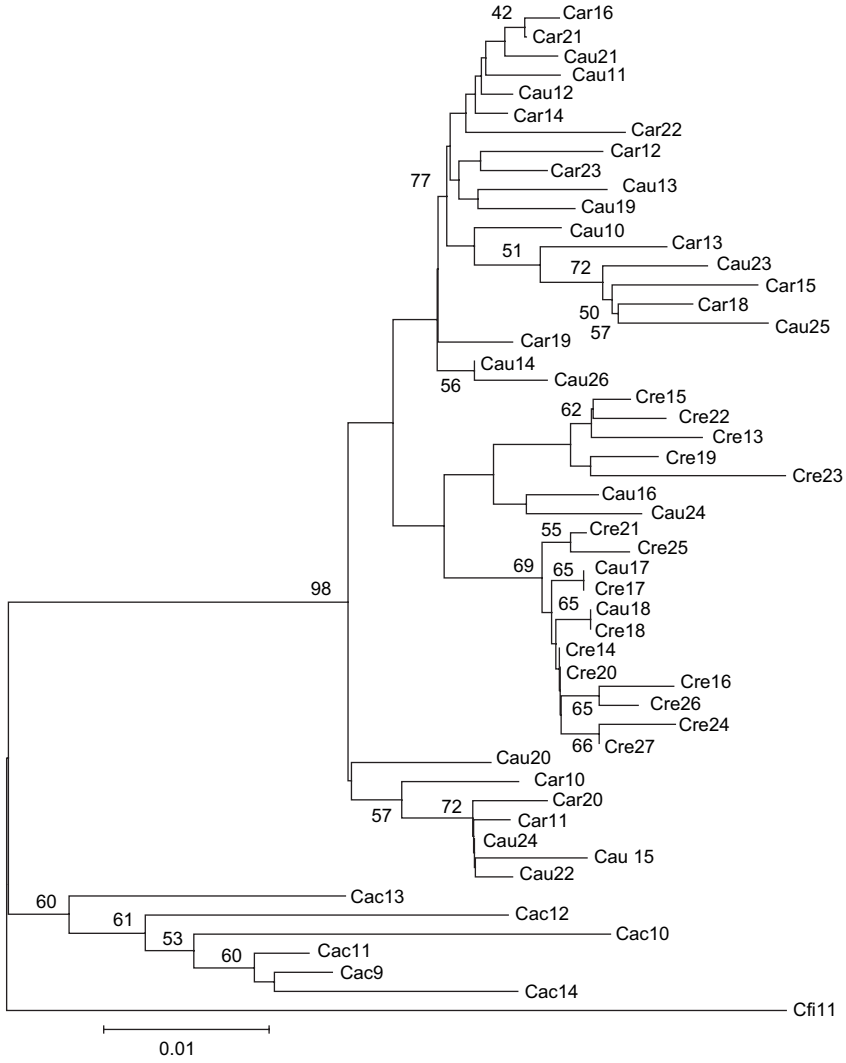

Figure 3. NJ tree of HKY distances among haplotypes in the subgenus *Xiphypops*, genus *Centropyge*. Bootstrap values superimposed on single NJ tree for the complete dataset. Abbreviations of species names are defined in Table 1.

cichlids (Nagl et al. 2000), 6.5%–8.8%/MY for Lake Malawi cichlids (Sturmbauer et al. 2001), 7%/MY for New World cichlids (Barluenga and Meyer 2004), 7.7%–16.8%/MY for Indo-Pacific tasselfishes (Polynemidae; Chenoweth and Hughes 2003), 15%–20%/MY for Indo-Pacific sardines (Clupeidae; Bowen and Grant 1997), and 33%–100%/MY for Indo-Pacific butterflyfishes (Chaetodontidae; McMillan and Palumbi 1995, 1997), a group that is the sister family to angelfishes (Pomacanthidae). We chose the approximation of 10%/MY between lineages (5×10^{-8} within lineages) based on preliminary sequencing trials in several fishes (including pygmy angelfishes) that show control region divergences about five times higher than the comparable divergences with mtDNA cytochrome *b*, a locus that has been calibrated in multiple bony fishes at about 2%/MY (Bowen et al. 2001; see also Bermingham et al. 1997). The rate of 10%/MY is a first approximation, but it is possible that the initial control region separations occur at a higher rate (McMillan and Palumbi 1997). In interpreting the results for pygmy angelfishes, it is appropriate to consider the influence of a more rapid clock. Under a rate of 20%/MY, coalescence times would drop by half, such that the initial invasion of the Atlantic would be estimated at about a quarter million years (instead of a half million years). Overall, we regard time estimates as approximations on the scale of geological epochs, as reflected in our conclusion of a late Pleistocene invasion of the Atlantic.

2. The phylogenetic analyses do not unambiguously identify the Indian Ocean as the source of Atlantic colonizers. The SH test did not distinguish between *Cac* and *Cfi* as outgroups, and the NJ phylogeny shows a near-tricotomy between the Indo-Pacific *Cfi*, the Indian Ocean *Cac*, and the three Atlantic species (Figure 3). However, we support the

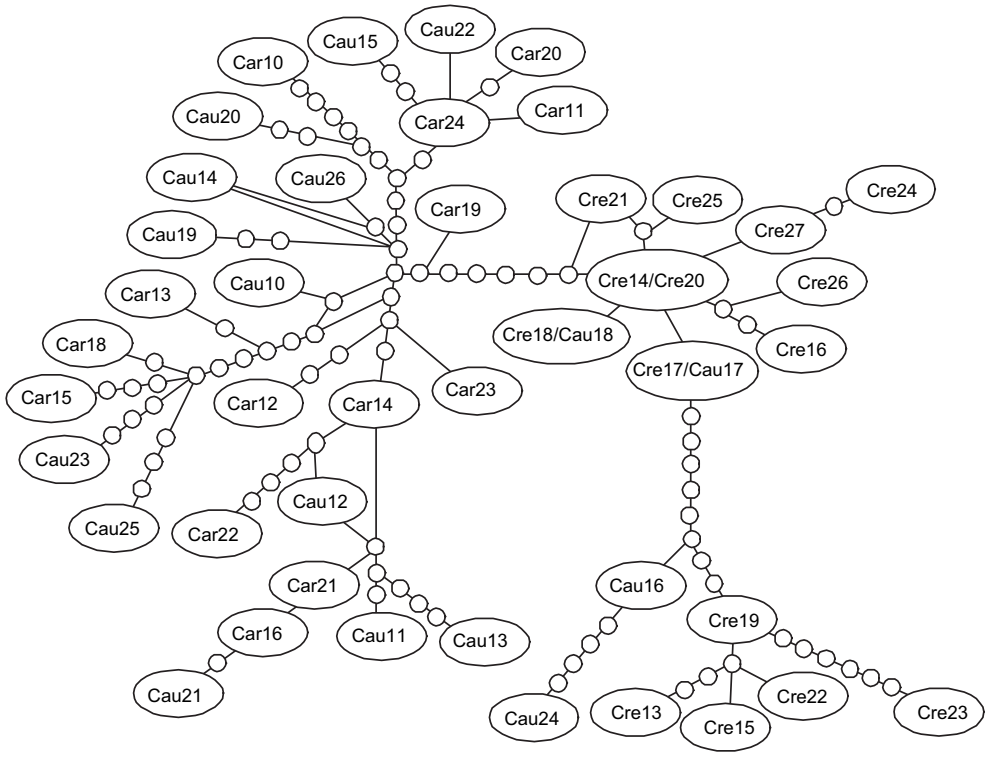

Figure 4. Statistical parsimony network for mtDNA haplotypes in three Atlantic species in the genus *Centropyge*. Open circles represent hypothetical intermediate haplotypes. Abbreviations of species names are defined in Table 1.

biogeographic scenario of an Indian Ocean source of Atlantic species based on four lines of evidence—(1) coloration: the Indian Ocean *Cac* is very similar in external appearance to the Atlantic species, (2) biogeography: *Cac* is the nearest geographic candidate because the genus *Centropyge* does not occur in the eastern Pacific (Robertson and Allen 2002), (3) timing: based on the broad range of molecular clocks proposed for the control region, the divergences here postdate the closure of the Isthmus of Panama, 3.1–3.5 Ma (Coates and Obando 1996), and (4) genetic distances: *Cac* is the closest relative to the Atlantic species ($d = 0.059$) compared with the Indo-Pacific *Cfi* ($d = 0.077$).

3. Here we define *Xiphypops* (sensu stricto) according to Pyle (2003), instead of the broader definition by Bellwood et al. (2004). In this classification, there might be a sixth member of the subgenus *Xiphypops*, which remains to be sampled; the cryptic *Centropyge* (*Xiphypops*?) *nigriocellus* occurs on shallow reefs of the Pacific. Although this species would be a desirable addition to our phylogeny, it is unlikely to change conclusions about Atlantic colonization and subsequent dispersal.

Incongruence between Taxonomic and Genetic Partitions

The incongruence between molecular and taxonomic partitions raises the question of whether the Atlantic *Centropyge* includes three valid species. Sequence divergence between the Caribbean *Car* and the Brazilian-Caribbean *Cau*, adjusted for within-species polymorphism, is $d_{adj} = 0.0$, and results of AMOVA are not significant; therefore, these putative species are not even distinguishable at a population level. The lack of molecular genetic differentiation is unexpected because the two types co-occur on coral rubble patches in the southern Caribbean and yet retain distinct color patterns with no known intermediates (Figure 1; Robertson DR and Rocha LA, personal observation).

Several possibilities may account for this incongruence. First, perhaps Atlantic *Centropyge* species would be more appropriately regarded as color variants of a single species. Color pattern polymorphisms have been demonstrated in other reef fishes, such as the Caribbean hamlet species complex (Genus *Hypoplectrus*, Family Serranidae), in which putative species distinguished by coloration are polyphyletic in mtDNA phylogenies (McCartney et al. 2003).

Second, populations of *Car* (Caribbean) and *Cau* (Brazil and southern Caribbean) may have been geographically isolated, but secondary contact in the southern Caribbean has resulted in introgression of mtDNA lineages. These two Atlantic taxa might be capable of hybridization, as has been documented for *Centropyge* species in the Indo-West Pacific (Steene 1978; Pyle and Randall 1994). However, we discount this hypothesis based on the mtDNA genealogy (Figure 2), which does not show distinct Brazilian and Caribbean lineages, with introgression of the Brazilian lineage into the Caribbean population. Topologies that indicate reconnections

Table 2. Estimates of historical and current demographic parameters based on fit of mtDNA control region mismatch distributions of mtDNA to population growth model in Atlantic and Indian ocean species of *Centropyge*

Species	Mean	Variance	τ	Years	Θ_0	N_f	Θ_1	N_f
Car	9.0	11.0	9.92	2.2×10^5	0.006	1	89.34	1.97×10^6
Cau	10.2	9.7	10.86	2.4×10^5	0.0	0.0	561.17	1.24×10^7
Cre	7.3	20.2	12.72	2.8×10^5	0.002	14.10	0.44	9.69×10^3
Atlantic	10.2	13.1	11.33	2.5×10^5	0.0	0.0	142.48	3.14×10^6
Cac	15.1	21.1	17.90	4.0×10^5	0.0	0.0	452.50	9.97×10^6

by long-isolated lineages can be found in other fishes, including the Indo-Pacific parrotfish *Chlorurus sordidus* (Bay et al. 2004). An examination of nuclear molecular markers is needed to test this hypothesis more rigorously.

A third explanation is that *Car* and *Cau* are valid species but have become reproductively isolated very recently, perhaps in the last few thousand years. In this scenario, the sorting of mtDNA lineages through random drift has not yet proceeded to the stage of reciprocal monophyly, a condition that can be expected in recently derived species (Bowen 1998; Campton et al. 2000; Rocha 2004). Brazil and the Caribbean are separated by 2300 km of inhospitable habitat, including mud bottom and freshwater outflows of the Amazon and Orinoco rivers, which represent a substantial barrier to reef-associated species (Briggs 1974; Muss et al. 2001; Rocha et al. 2002). Of course, this barrier must have been recently surmounted to account for the distribution of *Cau* on both sides of the Amazon. The high sea levels of the current interglacial allow a marine corridor under the freshwater Amazon outflow, which can be colonized by deepwater species (Rocha 2003). If *Cau*, *Cre*, and *Car* are valid species, the shallow pattern of sequence divergences and the recent coalescence of haplotypes indicate that reproductive isolation has ensued over a markedly brief interval.

Among these three possibilities, we endorse the first (color morphs of a single species) because the mtDNA gene genealogy does not indicate previously isolated lineages and most importantly because there are no characters beyond coloration that distinguish *Car* and *Cau* (Burgess 1974; Pyle 2003). In particular, the species-level distinction of *Cau* is based entirely on coloration, with a description in Tropical Fish Hobbyist (Burgess 1974), a highly respected magazine but not a peer-reviewed scientific journal. Although we regard Atlantic *Centropyge* as three color morphs, perhaps suitable for subspecific recognition, we caution against synonymizing these taxa on the basis of the mtDNA data. First, taxonomic nomenclature should be based on several lines of evidence and (more to the point) should not be revised on the basis of a single character system such as mtDNA. Second, the distributions of *Car*, *Cau*, and *Cre* are allopatric for the most part. Third, a test of hybridization in the area of overlap is lacking. To address species status, a morphological series should be initiated, nuclear DNA surveys would also be informative, field observation could test for assortative mating behavior, and captive breeding could address the issue of reproductive barriers. Frank Baesch (Reef Culture Technologies LLC, Oahu, HI) has raised *Centropyge* species through the larval stage in captivity, and this technology could directly address the hypothesis that Atlantic *Centropyge* are sibling species with recent reproductive isolation. Captive breeding could also resolve key issues concerning the inheritance of color patterns.

Table 3. Estimates of demographic parameters from coalescence analysis

Species	Θ_t	N_f	g^a
Car	1.27	1.27×10^7	754.7
Cau	1.12	1.12×10^7	497.5
Cre	0.21	2.1×10^6	466.5
Atlantic	1.35	1.35×10^7	416.5
Cac	1.78	1.78×10^7	271.0
Atlantic-Indian	0.59	5.9×10^6	209.9
Atlantic-Indian-Pacific	0.51	5.12×10^6	151.8

[a] Growth parameter in units of $1/\mu$ generations in the equation $\Theta_t = \Theta_0^{-gt}$, where $\Theta = 2N\mu$. t is the number of generations for one mutation to accumulate.

Incongruence between Coloration and Genetic Partitions

The dearth of morphological characters to distinguish species is a perennial problem in the taxonomy of *Centropyge* (Pyle R, personal communication) and other reef fishes, invoking the broader issue of whether coloration is a reliable indicator of evolutionary separations. Several recent cases indicate caution.

In a survey of West Atlantic wrasses (genus *Halichoeres*), Rocha (2004) found that color differences between Caribbean and Brazilian populations were matched by divergent mtDNA lineages (see also Rocha et al. 2005b). In these cases, the concordance of coloration and genetics was sufficient to resurrect species status for the Brazilian *H. dimidiatus* and *H. penrosei*. However, a striking color difference in *H. garnoti*, between populations of Bermuda and the Caribbean, was not matched by genetic differentiation.

In the Indo-Pacific damselfish (*Dascyllus trimaculatus*) species complex, Bernardi et al. (2002) found that two mtDNA lineages correspond to color morphs but three others do not. In this case, additional evolutionary units are masked by uniformity in coloration. In contrast, color morphs of the spiny damselfish (*Acanthochromis polyacanthus*) aligned well with genetic divergences and assortative mating behavior (Planes and Doherty 1997a,b)

In a survey of the Caribbean hamlets (genus *Hypoplectrus*), McCartney et al. (2003) observed reproductive isolation of

sympatric color morphs (albeit at a population-genetic level) in Puerto Rico but no significant separation among the same color morphs in Panama. This finding is especially notable in the presence of strong assortative mating among some but not all color morphs (Fischer 1980).

In the Indo-Pacific butterflyfishes (genus *Chaetodon*), McMillan et al. (1999) observed concordance between coloration and genetic partitions (allozymes and mtDNA) in one of three putative sister species. The other two color morphs, *C. punctatofasciatus* and *C. pelewensis*, showed no evidence of genetic differentiation or assortative mating behavior.

Clearly, it can be hazardous to define evolutionary (and taxonomic) distinctions by coloration in the absence of support from behavior, genetics, or morphology. However, the evolutionary significance of coloration cannot be underestimated. In many fishes, coloration is a key character for mate recognition and hence can be subject to strong sexual selection (Seehausen et al. 1997, 1999). In other cases, sympatric color morphs may show ecological differentiation in the absence of diagnostic genetic differences (Alesandrini and Bernardi 1999; Medioni et al. 2001). Hence, divergence in coloration is postulated to drive adaptive radiations in some of the most speciose reef fish families (Taylor and Hellberg 2005). Although no ecological partitions are documented among Atlantic *Centropyge* spp., Allen (1985) reported differences in anal fin coloration between sexes of *Cre*, providing a tentative perch to support such arguments. In these circumstances, it is possible that color differentiation will outstrip morphological and genetic differentiation and provide the first indication of emerging species (Bowen 1998; Streelman and Danley 2003).

Dispersal from the Indian Ocean

Most of the contemporary reef fish families were established by the lower Eocene (50 Ma), and modern genera were present by the mid-Miocene (10 Ma) (Bellwood and Wainwright 2002). Much of the Atlantic reef fauna predates the closure of the Tethys Sea, including the endemic lineages of parrotfishes (genus *Sparisoma*; Bernardi et al. 2000) and wrasses (genus *Halichoeres*; Barber and Bellwood 2005). Other separations came after the Tethys termination but before the closure of the Isthmus of Panama, including Atlantic wrasses (genus *Thalassoma*; Bernardi et al. 2004), trumpetfishes (genus *Aulostomus*; Bowen et al. 2001), blennies (genus *Ophioblennius*; Muss et al. 2001), and groupers (genera *Dermatolepis* and *Epinephelus*; Craig et al. 2001, 2004).

The genetic affinity of the Indian Ocean species *Cac* to the three Atlantic Ocean species indicates a more recent zoogeographic connection between the Indian and Atlantic oceans. The absence of *Centropyge* in the East Pacific (Robertson and Allen 2002) and the timing of the Atlantic colonization (postdating closure of the Isthmus of Panama about 3.1–3.5 Ma) also support a model of recent dispersal around southern Africa. Nonetheless, the cold Benguela Current off the west coast of southern Africa represents a formidable barrier to the dispersal of tropical fishes into the Atlantic (Gibbons and Thiebault-Botha 2002). About 3000 km separate the tropical reefs of southeastern Africa (Indian Ocean) from the warm waters off southwestern Africa (Atlantic Ocean). The colonization of the Atlantic may have been achieved by planktonic larvae transported westward in warm gyres that bud off from the Agulhas Current and become entrained in the northward-moving Benguela Current (van Ballegooyen et al. 1991; Penven et al. 2001). Estimates of the time to coalescence for the mtDNA control regions sequences examined in this study place *Centropyge* in the Atlantic at least 250,000 years before the present. In a companion study, Rocha et al. (2005a) demonstrate more recent colonization of the Atlantic by an Indo-Pacific reef fish (goby, genus *Gnatholepis*) on the order of 125,000 years before present. Several grouper species have ranges that span both sides of Africa (e.g., *Epinephelus marginatus*; Heemstra and Randall 1993), indicating recent dispersal around the tip of the African continent, although estimates of gene flow in these species are not available.

A likely scenario is that dispersal into the southwestern Atlantic was enhanced by an increase in Agulhas Current throughflow into the South Atlantic during hiatuses of Benguela upwelling that occurred at the end of each ice age (Chang et al. 1999; Peeters et al. 2004; Rocha et al. 2005a). Plankton in sediment cores from the southeast Atlantic indicate episodes of warming during the marine isotope stages 7, 9, and 11, which date to about 200,000, 300,000, and 400,000 years, respectively (Flores et al. 1999). Immediately following each of the ice ages, a spike of tropical plankton appears in sediment cores off southwestern Africa, indicating an increased flow of Agulhas Current water into the South Atlantic (Peeters et al. 2004). It is tempting to associate the date of mtDNA control region coalescence with the end of glacial Termination III at 240,000 years. However, the error on the estimate of coalescence time is large and includes both Terminations IV (430,000 years) and II (130,000 years).

The present-day geographic distribution of *Centropyge* in the Atlantic poses another zoogeographic question. Atlantic species of *Centropyge* occur in the tropical western and central Atlantic but have not been reported in the tropical eastern Atlantic. This distribution indicates a dispersal route into the Atlantic either to the Mid-Atlantic Ridge islands or directly to the shores of South America. Rafts of Indian Ocean algae have been observed at St. Helena, demonstrating dispersals to the Mid-Atlantic Ridge islands (Edwards 1990). These islands may therefore act as stepping-stone habitats for dispersal into the western Atlantic. Alternatively, dispersal directly into the western Atlantic may be possible in the warm South Equatorial Current (Gordon 2003), which flows northwest from Africa to the western Atlantic. Present-day currents cross the Atlantic from Africa to Brazil in about 70 days (Scheltema 1971), but this is about twice the 30- to 35-day larval duration estimated for species of *Centropyge* (Thresher and Brothers 1985).

The genetic data for Atlantic *Centropyge* support a model of direct dispersal from the Indian Ocean to Brazil because basal (ancestral) Atlantic haplotypes occur in the western Atlantic. Other groups of species, including mollusks (Vermeij and Rosenberg 1993), reef fishes (Rocha et al. 2005a), and

sea turtles (Bowen et al. 1994) show a similar zoogeographic pattern, indicating a general dispersal route into the western Atlantic from the Indian Ocean. Under this scenario, *Centropyge* inhabiting waters around the Mid-Atlantic Ridge islands must have originated from the western Atlantic and not directly from the Indian Ocean. The two haplotype clusters that include the mid-Atlantic *Cre*, each with significant bootstrap values, support a model of at least two dispersals to the Mid-Atlantic Ridge from the Brazilian population, probably mediated by the southern equatorial currents.

Prospectus

The mtDNA data for the subgenus *Xiphypops* resolve several questions about Atlantic colonization but present some challenges as well. In other surveys of West Atlantic reef fishes, most species show isolation between the Caribbean and Brazil, with a break in distribution at the Amazon Barrier. The *Centropyge* species are among the few that do not, an especially notable finding in view of species designations for Brazilian and Caribbean forms. However, incongruence between genetic partitions and coloration have recently been reported in a suite of reef fishes including wrasses, hamlets, damselfishes, and butterflyfishes. These findings may indicate that coloration is a fickle foundation for evolutionary and taxonomic distinctions. Alternately, these findings raise the possibility that color morphs subject to sexual selection or ecological differentiation (and lacking morphological or molecular diagnostics) are the raw edge of emerging species on coral reefs.

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