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Evolution of pygmy angelfishes: Recent divergences, introgression, and the usefulness of color in taxonomy

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ABSTRACT

The pygmy angelfishes (genus *Centropyge*, family Pomacanthidae) are brightly colored species that occupy reef habitats in every tropical ocean. Some species are rarely observed because they occur below conventional scuba depths. Their striking coloration can command thousands of U.S. dollars in the aquarium trade, and closely related species are often distinguished only by coloration. These factors have impeded phylogenetic resolution, and every phylogeographic survey to date has reported discordance between coloration, taxonomy, and genetic partitions. Here we report a phylogenetic survey of 29 of the 34 recognized species ($N = 94$ plus 23 outgroups), based on two mtDNA and three nuclear loci, totaling 2272 bp. The resulting ML and Baysian trees are highly concordant and indicate that the genus *Centropyge* is paraphyletic, consistent with a previous analysis of the family Pomacanthidae. Two recognized genera (*Apolemichthys* and *Genicanthus*) nest within *Centropyge*, and two subgenera (*Xiphypops* and *Paracentropyge*) comprise monophyletic lineages that should be elevated to genus level. Based on an age estimate of 38 Ma for the family Pomacanthidae, *Centropyge* diverged from the closest extant genus *Pygoplites* about 33 Ma, three deep lineages within *Centropyge* diverged about 18–28 Ma, and four species complexes diverged 3–12 Ma. However, in 11 of 13 cases, putative species in these complexes are indistinguishable based on morphology and genetics, being defined solely by coloration. These cases indicate either emerging species or excessive taxonomic splitting based on brightly colored variants.

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1. Introduction

Coral reefs are characterized by a diverse and colorful assemblage of fishes whose origin dates back to at least 50–70 Ma (Bellwood and Wainwright, 2002). Angelfishes (Pomacanthidae) are a conspicuous family of about 84 reef-associated species divided into seven genera (Allen et al., 1998 and Pyle, 2003). These vividly-colored fishes are found in tropical and subtropical latitudes across the globe and are differentiated from the closely related butterflyfishes (Chaetodontidae) by the presence of a prominent spine on the lower edge of the gill cover. *Centropyge* is the most species-rich genus of angelfishes, and is distinguished by the shape and spination of the preorbital surface as well as relatively small body size, the latter giving rise to their common name of pygmy angelfishes. Species in the genus, like most angelfishes, are protogynous hermaphrodites (Moyer, 1987). They are haremic, with males guarding the territories of one to several females. Observational data indicates that most species spawn around dusk, and some species are thought to mate year round (Bauer and Bauer, 1981). Due to their vibrant colors, these species, as well as other angelfishes, are highly valued in the aquarium trade with rare species selling for thousands of U.S. dollars.

The only modern revision of *Centropyge* recognizes 32 species based on morphology, geography, and coloration (Pyle, 2003). Two additional species have since been described (*Centropyge abei* Allen et al., 2006 and *C. deborae* Shen, Ho and Chang, 2012). Three subgenera are recognized based on preorbital spination, body shape, and coloration: *Centropyge* (26 spp), *Xiphypops* (5 spp),

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and *Paracentropyge* (3 spp) with the latter treated as a valid genus by some authors (Pyle, 2003). While morphological distinctions separate the subgenera, many of the putative species within these groups are distinguished solely by color, and often hybridize in areas of sympatry (Pyle, 2003; Hobbs et al., 2009 and DiBattista et al., 2012).

These circumstances led Pyle (2003) to suggest that *Centropyge* contains monophyletic groups of recently diverged species. Subsequent analyses have supported this suggestion, with three closely related species inhabiting tropical reefs of the Atlantic (Bowen et al., 2006), and genetic homogeneity among regional color morphs of the Flame Angelfish (*Centropyge loriculus*) of the Pacific (Schultz et al., 2007). In an Indo-Pacific species complex of three morphotypes with striking color differences (*C. flavissima/eibli/vrolikii*), genetic lineages correspond not to described species, but to geographic regions (DiBattista et al., 2012). In all molecular studies to date, discordance is apparent between coloration, genetics, and taxonomy. Concern is warranted, given that coloration is a primary foundation for taxonomy in this group.

In the first molecular evaluation of the Pomacanthidae (Bellwood et al., 2004), problems with the taxonomy of *Centropyge* became apparent, with suggestions that this genus is paraphyletic and may be part of a larger clade incorporating *Apolemichthys* and *Genicanthus*. Clearly a thorough evaluation of the genus was required.

Based on this backdrop of taxonomic, genetic, and biogeographic discordance, here we present sequence data from two mitochondrial (COI and cyt *b*) and three nuclear markers (TMO, RAG2, and S7) from 29 of the 34 recognized species in the genus plus representatives from five of the other six genera in the family. We address evolutionary relationships of the genus *Centropyge* with four purposes. First, we investigate the presumed paraphyly of this group, as reported initially by Bellwood et al. (2004). Second, we provide the first independent appraisal of the subgenus designations of Pyle (2003), which may influence taxonomic revisions. Third, we use a fossil-based molecular clock to examine the evolutionary and biogeographic history of *Centropyge*. Finally, we evaluate the extent to which the discordance noted above between taxonomy, biogeography, and phylogenetics is present across the genus or family. This could indicate that an emphasis on vivid coloration has yielded a misleading taxonomy.

2. Materials and methods

2.1. Sample collection and DNA extraction

Specimens were collected with pole-spears, hand-nets, and anesthetics (quinaldine and rotenone) while scuba diving. The collections of these species span 23 years, and include diving to the limits of conventional scuba, plus rebreather diving to 150 m with tri-mix gas (e.g., Pyle and Randall, 1992 and Allen et al., 2006). Details of these collections are provided in Table S1. Field identifications were made by authors and colleagues, and in most cases these were unambiguous based on coloration and geographic range information. In cases of suspected hybridization or range overlap between similar species, photo or specimen vouchers were taken by authors.

A total of 94 specimens across 29 of the 34 recognized species in the genus *Centropyge* were obtained for this study. The sister group to *Centropyge* is not known with certainty, therefore, an additional 23 specimens from five other genera in the family (Table S1) were analyzed. Tissues were preserved in either >70% ethanol (EtOH) or saturated salt-DMSO (Seutin et al., 1991) and stored at room temperature. Total genomic DNA was isolated using either a DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol or the modified "HotSHOT method" (Meeker et al., 2007) and subsequently stored at −20 °C.

2.2. PCR amplification and sequencing

We resolved 614 bp of the mitochondrial cytochrome oxidase I gene (COI) using the primers FishF2 and FishR2 of Ward et al. (2005) and 596 bp of the cytochrome *b* gene (cyt *b*) using L14725 of Song et al. (1998) and H15573 of Taberlet et al. (1992). We resolved 346 bp of the TMO 4C4 gene (TMO) using the primers TMO-4C4F and TMO-4C4R of Streelman and Karl (1997), 408 bp of the recombination-activating gene 2 (RAG2) using RAG2 F1 (5′-GAGGGCCATCTCCTTCTCCAA′) and RAG2 F3 (5′-GATGGCCTTCCCTCTGTGGGTAC′) and 308 bp of the first intron of the S7 ribosomal protein gene (S7) using S7RPEX1F and S7RPEX2R of Chow and Hazama (1998). In some cases amplification of the TMO 4C4 and RAG2 genes was achieved using the modified primers of DiBattista et al. (2012).

Polymerase chain reactions (PCRs) were carried out in a 10 µl volume containing 2–15 ng of template DNA, 0.2–0.3 µM of each primer, 5 µl of the premixed PCR solution BioMix Red™ (Bioline Inc., Springfield, NJ, USA), and deionized water to volume. PCR reactions utilized the following cycling parameters: initial denaturation at 95 °C and final extension at 72 °C (for 5 min each), with an intervening 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature (COI, TMO, RAG2 = 56 °C; cyt *b* = 54–58 °C; S7 = 58–60 °C), and 45 s at 72 °C. Amplification products were purified using 0.75 units of Exonuclease I and 0.5 units of Shrimp Alkaline Phosphatase (ExoSAP; USB, Cleveland, OH, USA) per 7.5 µl PCR products at 37 °C for 60 min, followed by deactivation at 85 °C for 15 min. DNA sequencing was performed with fluorescently-labeled dideoxy terminators on an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Hawai'i Advanced Studies of Genomics, Proteomics and Bioinformatics sequencing facility.

Sequences for each locus were aligned and edited using the DNA sequence assembly and analysis software Geneious Pro 5.5.6 (Drummond et al., 2011). Unique sequences were identified using the Haplotype Collapser and Converter option in FaBox v.1.35 (http://birc.au.dk/fabox) and deposited in GenBank (see Table S1 for accession numbers).

2.3. Phylogenetic analyses

All analyses were performed on the California Academy of Sciences Center for Comparative Genomics PhyloCluster. Previously published phylogenies suggested that angelfish are closely allied with butterflyfishes (Chaetodontidae; Bellwood et al., 2004), therefore, sequences from two butterflyfishes of the genus *Chaetodon* were obtained from GenBank and used to root the trees (Table S1). Additionally, sequences from the angelfish *Pomacanthus maculosus* (a representative of the putative earliest branching genus within the Pomacanthidae; Bellwood et al., 2004) were downloaded from GenBank and included in the analyses (Table S1). The optimal model of sequence evolution for each alignment was selected using the Akaike Information Criterion (AIC) in jModelTest (Posada, 2008). Phylogenetic relationships were assessed based on the concatenated alignment.

Maximum likelihood (ML) analyses were conducted using the Randomized Accelerated Maximum Likelihood (RAxML) software v7.3.0 (Stamatakis, 2006). The GTR model of nucleotide substitution with the Γ model of rate heterogeneity was implemented using a random starting tree and four discrete rate categories. All model parameters were estimated by RAxML and partitions were assigned according to gene region. Eight independent runs were performed and the best trees from the individual runs were

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