



Brief Communication

Angelfishes, Paper Tigers, and the Devilish Taxonomy of the *Centropyge flavissima* Complex

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Abstract

The pygmy angelfishes (genus *Centropyge*) provide numerous examples of discordance between color morphology, taxonomy, and evolutionary genetic lineages. This discordance is especially evident in the *Centropyge flavissima* complex, which includes three primary color morphs, three previously recognized species (*C. flavissima*, *Centropyge eibli*, and *Centropyge vrolikii*) and three distinct mitochondrial (mtDNA) lineages that do not align with species designations. Our previous research showed that the putative *C. flavissima* arose independently in the Indian and Pacific Oceans, and the three mtDNA lineages align with geography rather than species assignments. Here, we add 157 specimens to the previous dataset of 291 specimens, spread across a greater geographic range, to pinpoint the distribution of mtDNA lineages and color morphs. We found that the mtDNA lineages show remarkably strong geographic boundaries corresponding to the Indian Ocean, Central-West Pacific, and Central-South Pacific. We also test the validity of the “Black Tiger *Centropyge*” in the *C. flavissima* species complex, a taxonomic oddity that is restricted to shoals and atolls off the coast of northwestern Australia, and the newly named *Centropyge cocosensis* assigned to the *C. flavissima* lineage in the Indian Ocean. We conclude that the Black Tiger *Centropyge* is not a valid species but rather an intermediate between sympatric color morphs that correspond to the putative species *C. eibli* and *C. vrolikii*. Our greater sampling efforts also do not support the genetic distinctiveness of *C. cocosensis* given shared mtDNA haplotypes with the sympatric *C. eibli* and *C. vrolikii*, but instead we find conflicting lines of evidence concerning the taxonomy of this group. We urge caution and taxonomic restraint until the true nature of this species complex can be revealed.

Subject areas: Population structure and phylogeography; Molecular systematics and phylogenetics

Key words: color variation, coral reef fish, hybridization, mitochondrial DNA, pygmy angelfish, taxonomy

Discordance between genetic divergence and color-based taxonomy is well documented in the radiant fishes that occupy coral reefs, including butterflyfishes (Chaetodontidae; [Hobbs et al. 2013](#);

[DiBattista et al. 2015a](#)), angelfishes (Pomacanthidae; [DiBattista et al. 2012](#); [Gaither et al. 2014](#)), damselfishes (Pomacentridae; [Bernardi et al. 2002](#); [Drew et al. 2010](#); [Leray et al. 2010](#)) and groupers

(Serranidae; Ramon et al. 2003; Garcia-Machado et al. 2004). The maintenance of regional color differences in the face of substantial gene flow is usually explained by selection driven by predation, habitat preference, territoriality (butterflyfish: McMillan et al. 1999) or mate recognition (*Hypoplectrus* complex; Puebla et al. 2008; Holt et al. 2011). These color differences may also be snapshots of the early phases of speciation.

The pygmy angelfishes (genus *Centropyge*) provide striking examples of discordant taxonomy, coloration, and genetics. All previous phylogeographic studies on *Centropyge* show a misalignment of coloration and genetic partitions (Bowen et al. 2006; Schultz et al. 2007; DiBattista et al. 2012; Gaither et al. 2014), with morphological characters providing poor taxonomic resolution (Pyle and Randall 1994; Pyle 2003). In the Atlantic, three species (corresponding to the Caribbean, Brazil, and mid-Atlantic ridge) share mitochondrial (mtDNA) haplotypes and are distinguishable only at a population level (Bowen et al. 2006). In the central Pacific, three regional color morphs of the flame angelfish (*Centropyge loricula*) share mtDNA haplotypes and lack even modest population genetic distinctions (Schultz et al. 2007). In the West Pacific, three species in the *Centropyge flavissima* complex (hereafter the “*flavissima*” complex, which includes *Centropyge vrolikii* and *Centropyge eibli*), host three divergent mtDNA lineages that align with geography but not species designation (DiBattista et al. 2012). This incongruence is pervasive throughout the *Centropyge* phylogenetic tree (Gaither et al. 2014).

How do these angelfishes maintain color differences in the face of recent or ongoing gene flow? This question is especially puzzling given that *Centropyge* species have high dispersal ability (Thresher and Brothers 1985) and frequently hybridize (Pyle and Randall 1994; Hobbs and Allen 2014), a combination that would seem to diminish opportunities for differentiation. Hybridization is common in reef fishes (especially angelfishes; Montanari et al. 2016) and is concentrated at biogeographic borders where closely related species overlap (Hobbs et al. 2013). These suture zones provide opportunities to understand how hybridizing fishes maintain species integrity. Two tropical marine suture zones have been identified in the Indo-Pacific: Christmas Island and the adjacent Cocos-Keeling Islands in the eastern Indian Ocean (mixing of Indian and Pacific Ocean fauna; Hobbs and Allen 2014), which includes the south coast of Java and Bali in Indonesia (Kuitert 2002) and the Socotra Archipelago in the western Indian Ocean (mixing of Red Sea, Arabian Sea, and Indo-Pacific fauna; DiBattista et al. 2015b).

The “*flavissima*” complex includes three previously recognized species that are distinguished only by color (Figure 1). The lemonpeel angelfish (*C. flavissima*) has a disjunct distribution across the central Indo-Pacific, *C. eibli* is in the eastern Indian Ocean, and *C. vrolikii* is in the central Indo-West Pacific (Figure 2). The disjunct distribution of *C. flavissima*, with a gap in the range of more than 3000 km, is accompanied by subtle differences in coloration (Allen et al. 1998), which has prompted the description of the eastern Indian Ocean morph as a new species (*C. cocosensis*; Shen et al. 2016). All species combinations hybridize with each other in the narrow regions where their geographic ranges overlap (Pyle and Randall 1994; Hobbs et al. 2009; Hobbs and Allen 2014). Christmas and Cocos-Keeling Islands in the eastern Indian Ocean is the only place where all three co-occur (Hobbs and Allen 2014). The taxonomy of these three species was questioned by DiBattista et al. (2012), which identified three monophyletic mtDNA lineages in the complex, but as noted above, the lineages did not align with color-based taxonomy but instead with geography (DiBattista et al. 2012). A fourth form (the “Black Tiger

Centropyge”; Figure 1) has been recorded in a restricted region off of northwestern Australia (Rowley Shoals, Scott Reef, and Ningaloo Reef; Hobbs JPA, unpublished data), however, its taxonomic status and relationship with the other species remains unknown (Debelius et al. 2003).

In an effort to more finely resolve the “*flavissima*” complex, and the discordance between phenotype and genotype, we expand on the analysis of DiBattista et al. (2012) by providing greater geographic sampling for all three species. Our additional sampling focuses on the central Pacific Ocean, the Cocos-Christmas hybrid zone and includes the fourth color morph—the Black Tiger *Centropyge*. With increased sampling we address: 1) the geographic extent of the three mtDNA lineages identified by DiBattista et al. (2012), 2) whether the discordance between phenotype and genotype extends throughout the geographic ranges, 3) the mtDNA affiliation of *C. cocosensis* relative to other members of the complex, and 4) the taxonomic status of the Black Tiger *Centropyge*. Is the Black Tiger *Centropyge* an undescribed species restricted to reefs off the northwestern coast of Australia, another color variant within the existing “*flavissima*” complex or a hybrid between *C. eibli* and *C. vrolikii* (Debelius et al. 2003)?

Materials and Methods

Sample Collection

A total of 68 *C. flavissima*, 14 *C. eibli*, 71 *C. vrolikii*, and 4 Black Tiger *Centropyge* were collected at sites across the Indo-Pacific while scuba diving or snorkeling between 2012 and 2014 (Table 1). Here, we add these samples to the 271 *C. flavissima*, 6 *C. eibli*, and 14 *C. vrolikii* individuals sequenced in DiBattista et al. (2012). Tissue samples were preserved in saturated salt-dimethyl sulfoxide (Seutin et al. 1991). Total genomic DNA was extracted using the “HotSHOT” protocol (Meeker et al. 2007) and subsequently stored at -20°C .

Mitochondrial DNA Sequencing

A 575 base pair (bp) segment of the mtDNA cytochrome *b* (*cyt b*) gene was amplified using modified primers, which were designed for these species (CFLM_FOR: 5′ - TCCCTCCAACATTTTCAGCAT - 3′; CFLM_REV: 5′ - TCTGGATCTCCAAGCAGGTT - 3′). This gene was chosen for the following three reasons: 1) we have access to *cyt b* sequences for all previous samples, 2) *cyt b* sequences are representative of the three monophyletic lineages within the “*flavissima*” complex, and 3) additional mitochondrial (COI) and nuclear (RAG2, TMO, S7) genes did not provide additional resolution across a much broader spectrum of species within the genus (DiBattista et al. 2012; Gaither et al. 2014). For PCR conditions and sequencing see DiBattista et al. (2012). All sequences were aligned, edited, and trimmed to a common length using Geneious Pro 4.8.4 (Drummond et al. 2009). The DNA sequences underlying these analyses have been deposited with NCBI (GenBank accession number KX773301–KX773457).

The evolutionary relationship among all angelfish haplotypes was further explored with a median-joining network constructed with the program PopArt 1.7 (<http://popart.otago.ac.nz>) using default settings.

Results and Discussion

Genetic analyses of the “*flavissima*” complex from across the Indo-Pacific confirm that these individuals partition into three reciprocally



Figure 1. “Pure” *Centropyge flavissima* (a, Pacific Ocean morph, Marshall Islands; b, Indian Ocean morph, Christmas Island, Australia), *Centropyge eibli* (c, Indonesia), and *Centropyge vrolikii* (d, Indonesia), in addition to some hybrids between the species (e, *C. flavissima* × *C. eibli*, Christmas Island, Australia; f, *C. flavissima* × *C. vrolikii*, Guam; g, *C. vrolikii* × *C. eibli*, Indonesia), and the “Black Tiger *Centropyge*” (h, Rowley Shoals). Note the characteristic blue ring around the orbit of the Pacific Ocean *C. flavissima* specimen (a), which is absent from the Indian Ocean *C. flavissima* specimen (b) (see online color version of article). Photo credits: Luiz Rocha (a, b, c, d, f, g), Tane Sinclair-Taylor (e), and Rudie Kuitert (h).

monophyletic mtDNA lineages, but the increased sampling in this study also reveals strong geographic structuring of these lineages and low levels of admixture between regions. As demonstrated in Figures 2 and 3, these lineages are grossly misaligned with color-based taxonomy, but instead show strong geographic cohesion into three regions: Indian Ocean, Central-West Pacific, and Central-South Pacific. Genetic analyses reveal that the Black Tiger *Centropyge* is not a distinct lineage; rather it is part of the Central-West Pacific lineage. In subsequent discussion, we use taxonomic designation to refer to the coloration-based species identities.

The Central-West Pacific lineage (i.e., lineage 1 in Figure 2b) is the most widespread and extends from the eastern Indian Ocean (Rowley Shoals and Scott Reef) eastward to Marquesas, north to Japan, and south to Middleton Reef, Australia. This lineage contains *C. vrolikii* and *C. flavissima* (Pacific morph only), as well as the

putative Black Tiger *Centropyge* (Figures 2 and 3). Notably, this lineage only includes two *C. eibli*, both sampled at the Cocos-Christmas hybrid zone, presumably sharing genes because of local introgression. The extensive sharing of haplotypes between *C. vrolikii* and Pacific *C. flavissima* most likely reflects greater introgression because these species hybridize in many locations where they co-occur around the range edge of *C. vrolikii* (e.g., Christmas Island, Vanuatu, Middleton Reef, Coringa, and Herald Cays in the Coral Sea, Guam, Kosrae, Pohnpei, Ryukyus Archipelago, Marshall Islands, and Ulithi; Pyle and Randall 1994; Hobbs et al. 2009; Pratchett et al. 2011; DiBattista et al. 2012; Hobbs and Allen 2014; Hobbs JPA, unpublished data; Allen G, personal communication). The geographic distribution of this lineage broadly resembles the geographic distribution of *C. vrolikii*. If hybridization between these two species results in unidirectional introgression of the maternally inherited

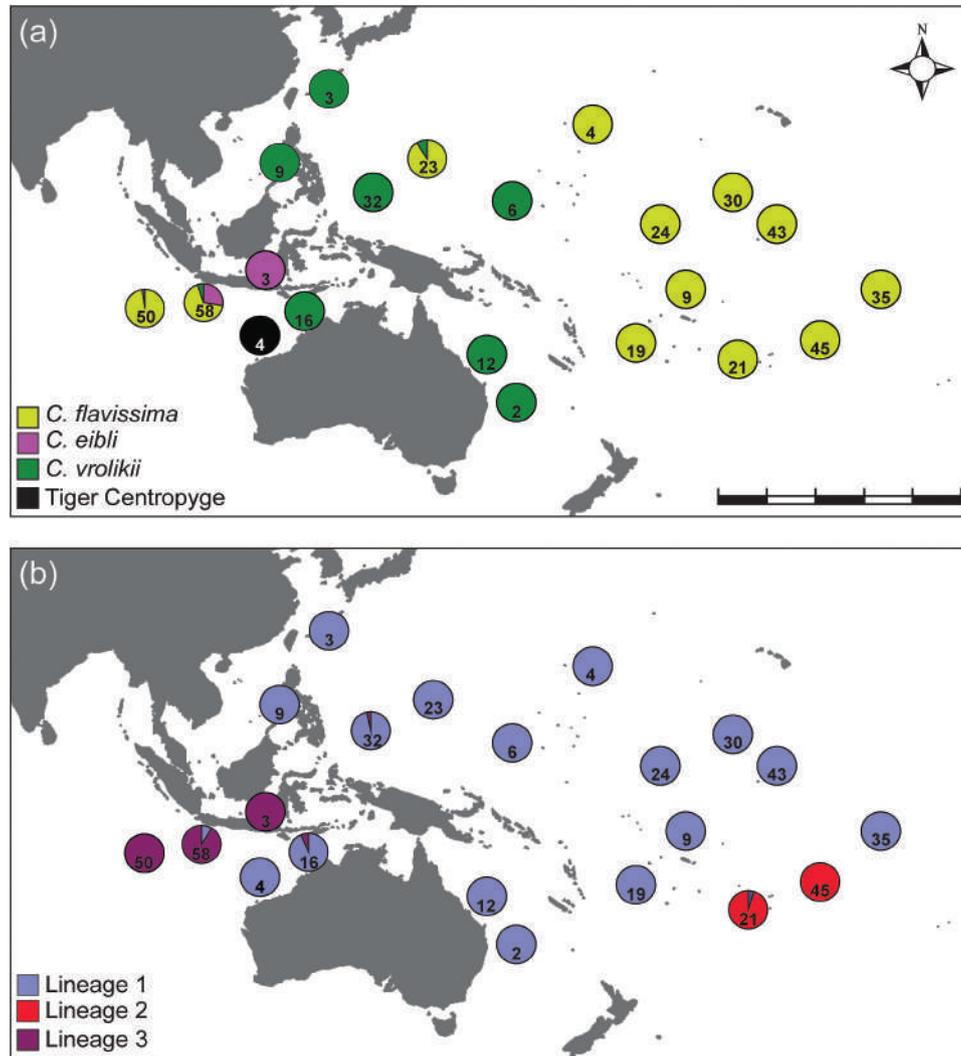


Figure 2. Indo-Pacific map showing distribution of individuals from the *Centropyge flavissima* complex based on (a) color morphology (see embedded key; *C. flavissima* = yellow, *Centropyge eibli* = dark pink, *Centropyge vrolikii* = dark green, “Black Tiger *Centropyge*” = black) (see online color version of article) and (b) mitochondrial DNA cytochrome *b* lineages (575 base pairs) as defined in Figure 1. Values within each circle indicate sample size per location.

mtDNA (as occurs in other hybridizing fishes; e.g., van Herwerden et al. 2006), then this Central-West Pacific group may be composed entirely of *C. vrolikii* mtDNA with *C. flavissima* mtDNA lost from the region. Thus, hybridization, unidirectional introgression, and dispersal out of the hybrid zone would explain why *C. vrolikii*, *C. flavissima*, and in rare cases *C. eibli* share the same haplotypes. Within the Central-West Pacific lineage, *C. flavissima* from the Marquesas Islands (Nuku Hiva) are genetically distinct from the rest of the lineage. Indeed, none of the 35 Marquesan specimens share haplotypes with any other site, perhaps indicating that this population is on the road to separation (DiBattista et al. 2012; Shen et al. 2016). This archipelago is at the eastern edge of the range and its isolation has been linked to peripheral speciation in other reef fishes (e.g., Planes and Fauvelot 2002; Gaither et al. 2010; Leray et al. 2010; Szabo et al. 2014; Gaither et al. 2015; Bowen et al. 2016).

In the east, the Central-South Pacific lineage (i.e., lineage 2 in Figure 2b) includes Rarotonga (Cook Islands) and Moorea (Society Islands), which are separated by 1100 km. The region hosts one member of the species complex (*C. flavissima*) almost exclusively, and these locations represent the furthest we have sampled from

the ranges of *C. vrolikii* and *C. eibli* (Figure 2). Thus, there is limited opportunity for *in situ* hybridization between species and long distance dispersal of introgressed individuals from hybrid zones to these sites. The Central-South Pacific lineage may therefore represent the “pure” genetic identity of *C. flavissima* based on the apparent lack of mixing.

The western most lineage (i.e., lineage 3 from Figure 2b) encompasses parts of Indonesia, Christmas Island, and the Cocos-Keeling Islands. This lineage includes *C. eibli* and *C. flavissima* (Indian Ocean morph only) individuals, but only rarely *C. vrolikii* (two individuals, one from Scott Reef and one from Palau) (Figure 2a). This most likely is the *C. eibli* lineage, and the occurrence of *C. flavissima* and *C. vrolikii* individuals in this lineage is due to hybridization and introgression at Bali, Christmas Island, and the Cocos-Keeling Islands. *Centropyge eibli* also occurs in Sri Lanka, Maldives, and India, future efforts should reveal the genetic affiliation of these sites.

The three lineages occur in distinct geographic ranges, however, there are a small number of exceptions (Figure 2b). One individual of the Indian Ocean (lineage 3) was detected in Palau, over 3000 km from the known range of the Indian Ocean lineage. One individual

Table 1. Sample size and location for individuals from the *Centropyge flavissima* complex

	<i>Centropyge eibli</i>	<i>Centropyge flavissima</i>	<i>Centropyge vrolikii</i>	'Tiger <i>Centropyge</i> '
Sample site				
Cocos-Keeling, Australia	1	10 (39)		
Christmas Island, Australia	13 (3)	12 (27)	3	
Rowley Shoals, Australia				4
Scott Reef, Australia			16	
Indonesia	(3)			
Okinawa, Japan			(3)	
Philippines			9	
Republic of Palau			31 (1)	
Guam, Northern Mariana Islands		21	2	
Pohnpei, Caroline Islands			(6)	
Phoenix Islands		(24)		
Wake Island		4		
Fiji		(19)		
Rarotonga, Cook Islands		21		
Moorea, Society Islands		(45)		
Nuku Hiva, Marquesas Islands		(35)		
Tokelau Islands		(9)		
Kiritimati, Line Islands		(43)		
Palmyra Atoll		(30)		
Great Barrier Reef, Australia			8 (4)	
Middleton Reef, Australia			2	

Taxonomic classifications are based on color morphology. Numbers represent new samples used in this study (GenBank accession numbers: KX773301–KX773457), whereas the numbers in parentheses represent samples from DiBattista et al. (2012) additionally included here (GenBank accession numbers: JQ914310–JQ914394).

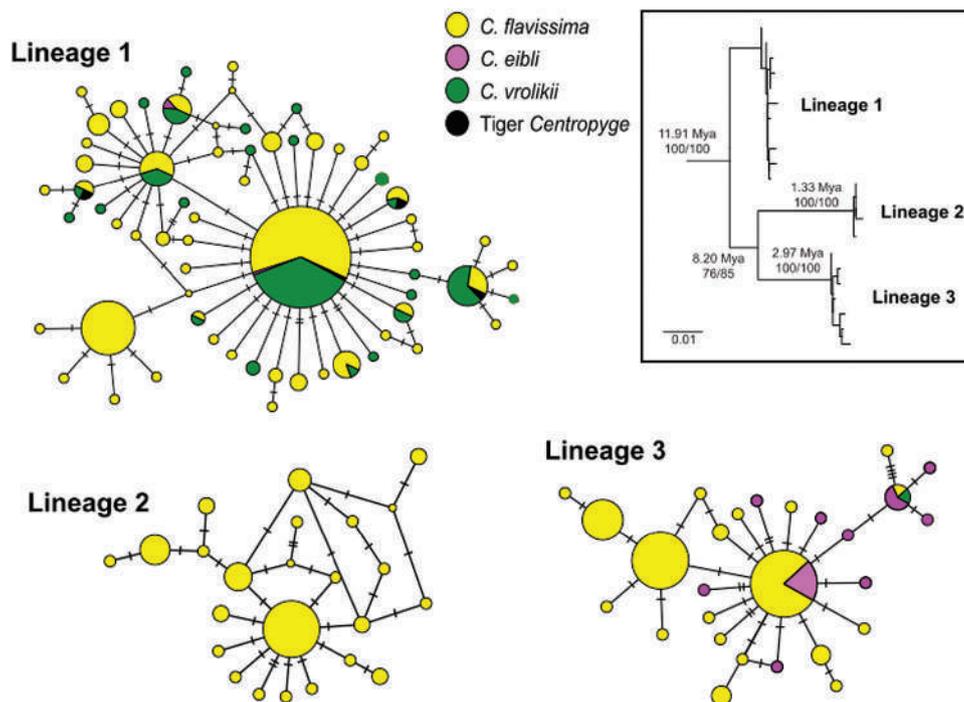


Figure 3. Median-joining networks of three divergent lineages from the *Centropyge flavissima* complex based on 575 bp of mitochondrial DNA cytochrome *b*. Each circle represents a unique haplotype and the size of the circle is proportional to its total frequency. Crossbars represent a single nucleotide change; colors denote species defined using color morphology (see online color version of article). Inset is from Gaither et al. (2014) and shows maximum likelihood tree for the “*flavissima*” complex. Above nodes are estimated ages in millions of years (Mya) based on analyses in BEAST, maximum-likelihood bootstrap support, and Bayesian posterior probabilities. Lineage 1 corresponds to the Central-West Pacific, lineage 2 corresponds to the Central-South Pacific, and lineage 3 corresponds to the Indian Ocean.

from the Central-West Pacific (lineage 1) was found in the South Pacific (Rarotonga). Five individuals (three *C. vrolikii*, one *C. eibli*, and one *C. flavissima*) from the Central-West Pacific (lineage 1) were also found at Christmas Island in the Indian Ocean. Some of these exceptions may represent individual long-distance dispersal events by larvae, stepping-stone dispersal across multiple generations, or remnants of a largely swamped mtDNA lineage.

The distinct coloration and peripheral location (Scott Reef and Rowley Shoals) of the Black Tiger *Centropyge* led to the initial expectation that it may be a separate species (Debelius et al. 2003; Figure 1). However, based on our analyses, the Black Tiger *Centropyge* may simply be another color morph of the Central-West Pacific lineage. Our four Black Tiger specimens nest within the lineage that contains most of the *C. vrolikii* individuals (lineage 1 from Figures 2 and 3). Although the Black Tiger *Centropyge* closely resembles *C. eibli* in coloration, it is not in the Indian Ocean lineage that contains the majority of *C. eibli*, but rather in the Central-West Pacific lineage that is composed mainly of *C. vrolikii* and *C. flavissima*. This is not surprising given that the Black Tiger *Centropyge* is found on reefs off north-western Australia, which are considered western outposts of Central-West Pacific fauna. Indeed, several other Central-West Pacific reef fishes also exhibit unique color morphs at these reefs (Debelius et al. 2003). Furthermore, at Scott Reef in particular, there are individuals of the Black Tiger *Centropyge*, *C. vrolikii*, and a range of intermediate color morphs indicating that the Black Tiger *Centropyge* and *C. vrolikii* freely interbreed (Figure 1); hence the Black Tiger is not a distinct species, but rather a “paper tiger.”

The discordance between genotype and phenotype in the “*flavissima*” complex means that each recognized species contains multiple mtDNA lineages and all three previously recognized species include lineage 3. Two examples highlight this discordance. First, *C. flavissima* coloration is completely different from other members of the complex (Figure 1), and yet it can be found in all three genetic lineages. Secondly, all three species co-occur at Christmas Island, where all specimens contain lineage 3. Extensive hybridization and introgression can explain both of these patterns but it is more difficult to explain how the three genetic lineages retain geographic integrity and how the strikingly different colors of the nominal species are maintained (Figure 1). These geographic partitions could indicate that individuals from one lineage cannot disperse to, colonize, or survive in the range of another lineage. Occasionally, two lineages co-occur at a location (see above), with one lineage being rare, and so these individuals may represent long distance dispersal events. The genetic signature of these rare “foreign” individuals may be lost through interbreeding with the local lineage and unidirectional gene flow (introgression), or these individuals may fail to reproduce. The maintenance of the distinct color patterns of the nominal species may reflect sexual or natural selection against intermediate phenotypes (DiBattista et al. 2012), which may extend to some of the other complexes in this genus (Gaither et al. 2014).

Phylogeographic studies have revealed many cases of sister species, or intraspecific evolutionary partitions, concordant with the geographic distinction of Pacific and Indian Ocean fauna (e.g., Gaither and Rocha 2013; Ahti et al. 2016; Bowen et al. 2016; Waldrop et al. 2016). In light of this common evolutionary pattern, it makes sense that the lemonpeel angelfish could be two distinct species. Certainly the two forms have allopatric distributions, coloration differences as illustrated by Shen et al. (2016), and evolutionary genetic distinctions as revealed by DiBattista et al. (2012) and the present study. The problem with this taxonomic conclusion is that in each case, the

lemonpeel angelfish appears to be highly introgressed (and shares haplotypes) with other species, particularly *C. vrolikii* and *C. eibli* in the Pacific Ocean and Indian Ocean, respectively. In light of these results, we urge caution and restraint in the taxonomic revision of this group of angelfishes. We acknowledge the effort made by Shen et al. (2016) to designate *C. cocosensis* as a valid species based on the coloration differences of these fish and the geographic separation (>3000 km) from the Pacific population, but we also cannot rule out that the putative species in the Indian Ocean is simply a yellow color morph of *C. eibli*. Indeed, the mtDNA presented here and nuclear DNA data of DiBattista et al. (2012) do not support the distinction between *C. cocosensis* and *C. eibli*. Using six specimens of *C. cocosensis* and four *C. eibli*, Shen et al. (2016) found no shared mtDNA haplotypes between the two putative species, but this was not the case in our much larger dataset from the Indian Ocean (49 *C. flavissima* and 19 *C. eibli*). Furthermore, DiBattista et al. (2012) found shared alleles at all three nuclear loci among a subset of these samples. This extensive sharing of haplotypes among color morphs, coupled with intermediate coloration and observed interbreeding at suture zones (e.g., Hobbs and Allen 2014), indicates hybridization and successful introgression. This also makes it difficult to find “pure” individuals to designate as a *C. cocosensis* holotype. *Centropyge cocosensis* may prove to be a valid species, but the genetics do not yet support this designation.

We forward the idea that traditional taxonomic approaches may not apply here. High-density genome scans using technologies such as restriction site-associated DNA sequencing (RADSeq; Andrews et al. 2016) may provide better resolution and reveal loci that are linked to natural or sexual selection processes. To confidently resolve this group, genome level molecular data coupled with a detailed knowledge of their ecology and elements of mate choice within their harem system are needed. Only then can we extend our genetic interpretation to understand the devilish taxonomy of this pygmy angelfish complex.

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