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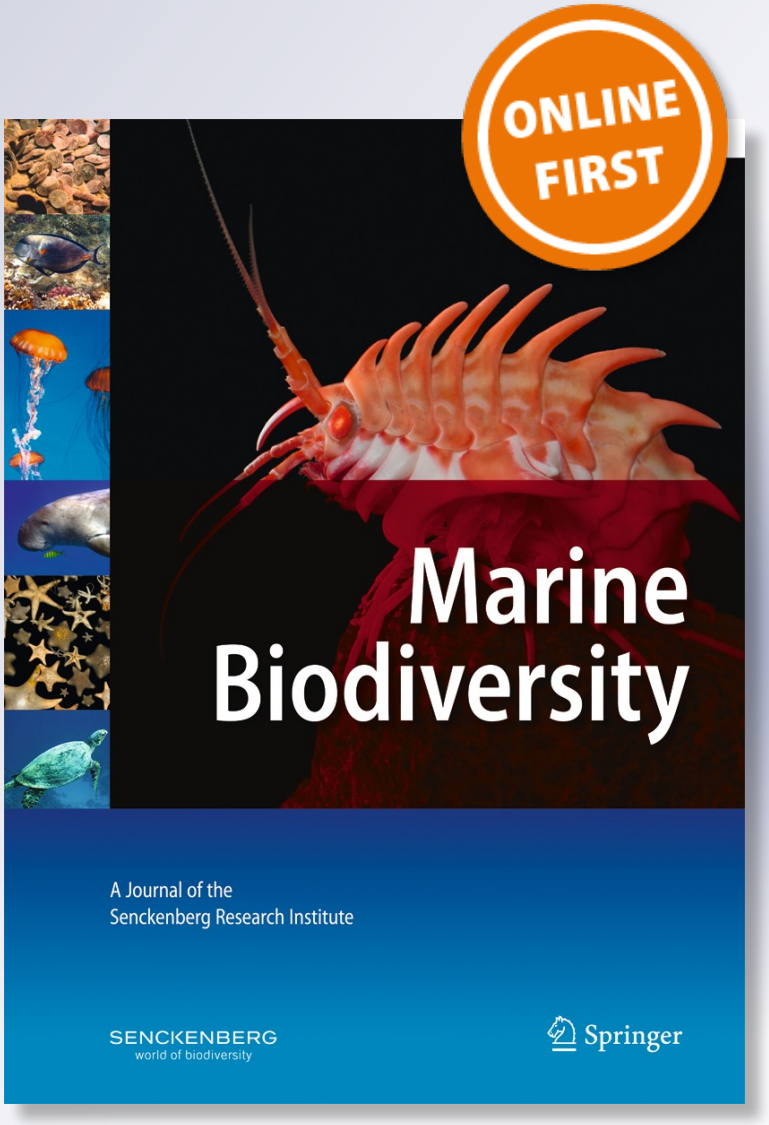

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ORIGINAL PAPER

Lemonpeel (*Centropyge flavissima*) and yellow (*C. heraldi*) pygmy angelfishes each consist of two geographically isolated sibling species

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Abstract Genetic variation was examined in two complex cases of Indo-Pacific pygmy angelfishes (genus *Centropyge*; Pomacanthidae). The lemonpeel pygmy angelfish *C. flavissima* (Cuvier and Valenciennes) has a geographically disjunct Indian vs. Pacific distribution and the individuals from these two regions differ by their colour patterns. Previous research on *C. flavissima* has shown mitochondrial introgression from two related species, *C. eibli* in the eastern Indian Ocean and *C. vrolikii* in the Pacific Ocean. Using the *16S* rDNA and the *CO1* gene as phylogeographic markers, we found no mitochondrial haplotypes in common between Indian Ocean *C. flavissima* and *C. eibli*, confirming partial genetic isolation, albeit recent. Also, we found substantial genetic differences between Indian and Pacific *C. flavissima* populations at the nuclear *ETS-2* intron locus. The Indian Ocean form of *C. flavissima*, thus geographically isolated by >2000 km distance from its Pacific Ocean counterpart, is described as a new species, *Centropyge cocosensis* sp. nov. *Centropyge cocosensis* sp. nov. differs in appearance from *C. flavissima* in having a conspicuous blue iris and a fainter, bluish eye ring. We also found that the yellow pygmy angelfish *C. heraldi* Woods and Schultz consists of two genetically distinct entities, one distributed widely in the northern tropical Indo-West Pacific, the other distributed in the southern Pacific Ocean. The name originally given to the blackfin pygmy angelfish, *C. woodheadi* Kuiter, is here resurrected to designate the latter.

Keywords Indo-West Pacific · Colour patterns · Molecular taxonomy · Mitochondrial introgression

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Kang-Ning Shen and Chih-Wei Chang contributed equally to this work.

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Introduction

Molecular genetics offers the tools and concepts to address the problem of cryptic species (Knowlton 2000) and increasingly provides evidence to new species discoveries in fishes (e.g. Borsa et al. 2010, 2013a, b, 2014; Baldwin et al. 2011; Liu et al. 2013; Puckridge et al. 2013; Delrieu-Trottin et al. 2014; Durand and Borsa 2015). Examples of geminate coral reef fish species between the Indian Ocean and the Pacific Ocean have been reported (Randall 1999; Woodland 1990, 1999; Craig et al. 2011; Borsa et al. 2014; Woodland and Anderson 2014). Genetic differentiation and subsequent speciation are thought to have been caused by the Indo-Pacific barrier, which extends from the Sunda Shelf to the Sahul Shelf (Rocha et al.

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2007). During glacial periods, connectivity between reef fish populations from either ocean was hampered: coral reef habitat suitable for adults was restricted due to lower sea level and cool upwelling caused by increased land mass, and conditions suitable for larval life were affected by lower salinity and higher turbidity caused by the discharge of large rivers (Fleminger 1986). These geminate or sister Indian Pacific fish species are morphologically similar but generally differ subtly by their colour patterns (e.g. Craig et al. 2011; Borsa et al. 2014; Woodland and Anderson 2014). Colour patterns in vertebrates are presumed to be subject to intense sexual selection, hence are likely to play an essential role in speciation (Endler et al. 2005). Following initial divergence in allopatry, reproductive isolation may have been reinforced (Butlin et al. 2012), despite secondary gene flow after the populations initially separated by the Indo-Pacific barrier have, once again, expanded and overlapped. However, geographic segregation of colour morphs may not necessarily be accompanied by parallel phylogeographic divergence, as inferred from the available genetic markers (Schultz et al. 2007, and references therein).

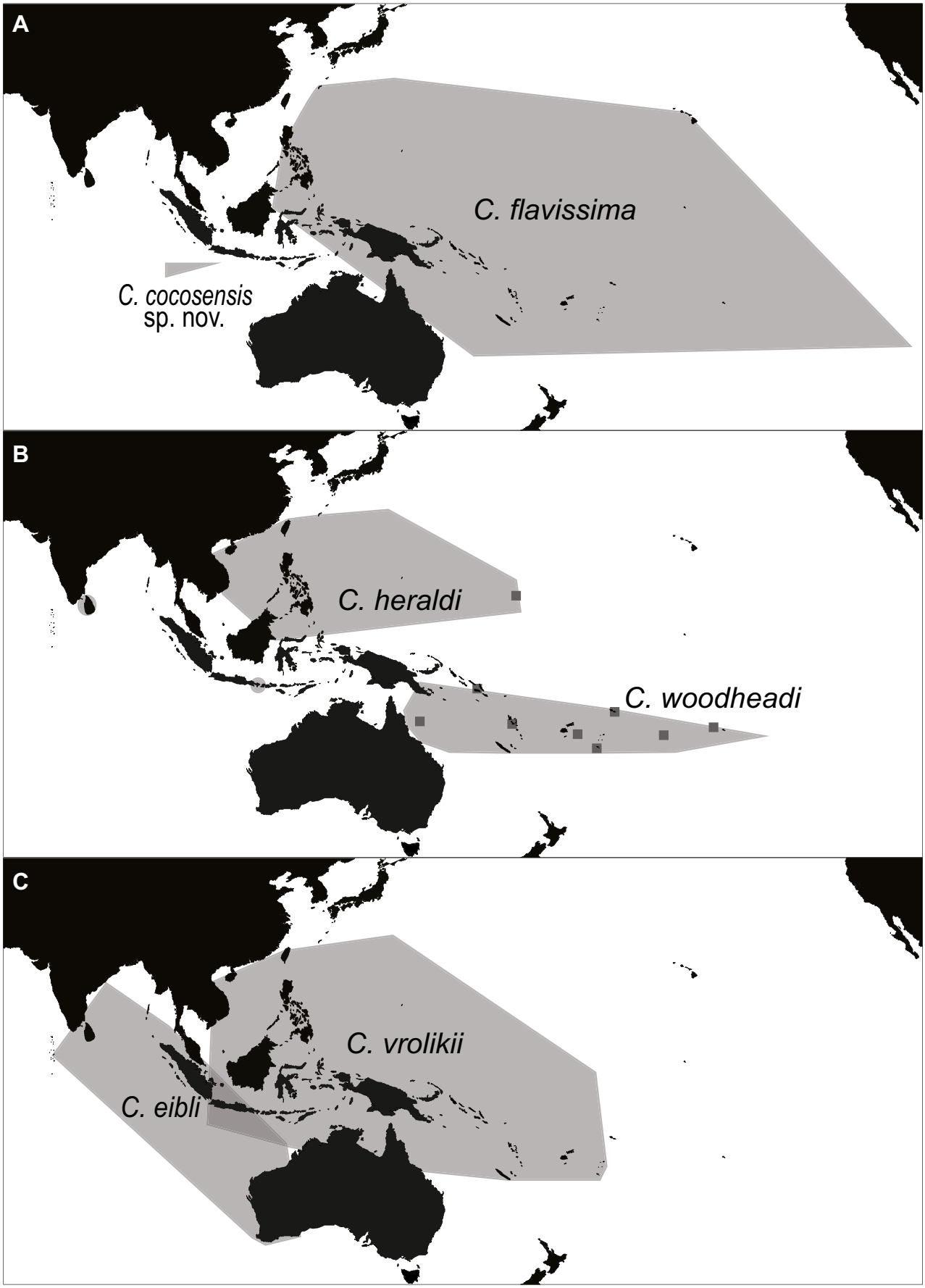
Pygmy angelfishes (genus *Centropyge*; Pomacanthidae) are generally colourful reef fishes. Species in this genus are mostly distinguished by their colour patterns, as meristics and morphometrics usually fail to provide diagnoses (Pyle 2003). While in some cases among Pomacanthidae sister species distinguished by colour patterns have proven to be genetically distinct (Randall and Rocha 2009; Shen et al. 2012), in other cases, mitochondrial haplotypes of different species within a species complex did not show genetic segregation (Bowen et al. 2006; Rocha et al. 2007) and, remarkably, distinct colour morphs within another species possessed the same mitochondrial haplotypes (Schultz et al. 2007).

We examined the genetic structure of two Pacific *Centropyge* species, the lemonpeel pygmy angelfish *C. flavissima* (Cuvier and Valenciennes, 1831) and the herald or yellow pygmy angelfish *C. heraldi* Schultz and Woods, 1953 (Schultz et al. 1953). *Centropyge flavissima* is one of the popular angelfishes in the ornamental fish trading market. It is characterised by blue eye ring and blue-margined gill operculum, spine and fins. Its geographic distribution ranges across the western and central Pacific and in the Cocos (Keeling) and Christmas Islands in the northeastern Indian Ocean (Hobbs et al. 2013; Fig. 1). Individuals of the latter population are similar in colour patterns to Pacific Ocean *C. flavissima* but they have a distinctive blue iris and a fainter bluish eye ring (Fig. 2a, b). It has been emphasised that the population in the northeastern Indian Ocean is isolated by more than 2000 km from the Pacific Ocean populations (Hobbs et al. 2013; Hobbs and Allen 2014). Based on distinct colour patterns and geographic isolation, Allen et al. (1998) hypothesised that the Indian Ocean and Pacific Ocean

Fig. 1 Geographic ranges [grey areas, being the polygons obtained by joining the most peripheral occurrence points reported in FishBase point maps (Froese and Pauly 2012)] of pygmy angelfishes of the *Centropyge flavissima* species complex. Background map of the Indo-West Pacific from Digital Vector Maps, San Diego (http://digital-vector-maps.com/). **a** *Centropyge cocosensis* sp. nov. and *C. flavissima*. **b** *Centropyge heraldi*. *Grey ellipses*: Sri Lanka (Steinke et al. 2009), Bali (present work); *dark grey squares*: localities where the blackfin form of *C. heraldi* (*C. woodheadi* sensu Kuiter, 1998) has been reported; from West to East: Holmes Reef, Solomon Islands, Vanuatu, Kwajalein, Maloa (type locality), Tonga, American Samoa, Cook Islands and Huahine (Randall 1997; Kuiter 1998; Randall and Carlson 2000; Steinke et al. 2009). The distribution of *C. heraldi* in the Pacific (as from Froese and Pauly 2012) includes the *woodheadi* form without distinction. **c** *Centropyge eibli* and *C. vrolikii*

populations of lemonpeel pygmy angelfish represent separate species. This differs from the view promoted by DiBattista et al. (2012), who reported that the Indian Ocean *C. flavissima* is genetically indistinct from the distinctly coloured blacktail angelfish *C. eibli* Klausewitz, 1963, while noting that some Pacific Ocean *C. flavissima* populations are genetically indistinct from the pearlscale angelfish *C. vrolikii* (Bleeker, 1853), the sister species of *C. eibli* (Fig. 3; DiBattista et al. 2012). The Indian Ocean form hybridises with *C. eibli*, while the Pacific Ocean form similarly hybridises with *C. vrolikii* (DiBattista et al. 2012). Three hypotheses may explain the paraphyly of *C. flavissima* haplotypes with *C. eibli* and *C. vrolikii* in the phylogeny of DiBattista et al. (2012) (Fig. 3): (1) incomplete lineage sorting among recently diverged species, (2) introgression or (3) different colour morphs within a single species (Gaither et al. 2014). The age of the mitochondrial lineages in the *C. flavissima* species complex (3.5–4.2 MYR; Gaither et al. 2014) tends to exclude hypothesis (1), whereas the observation of *C. flavissima* × *C. eibli* and *C. flavissima* × *C. vrolikii* hybrids (DiBattista et al. 2012; Hobbs and Allen 2014) supports hypothesis (2). However, few haplotypes are shared between Indian Ocean *C. flavissima* and *C. eibli* (DiBattista et al. 2012: their Figs. 4c and 5a), indicating partial reproductive isolation. A third lineage in the species complex characterises *C. flavissima* from Moorea in the geographically remote Society archipelago (Fig. 3). *Centropyge vrolikii* being absent from the Society archipelago (Randall 2005), the distinct *C. flavissima* lineage sampled in Moorea is thought to be a relict of the original lineage (DiBattista et al. 2012; Gaither et al. 2014).

The taxonomy of the Indo-Pacific yellow pygmy angelfish *C. heraldi* has also been subject to dispute. The distinctly coloured *C. woodheadi* Kuiter, 1998 was described recently, but was quickly synonymised with *C. heraldi* (Randall and Carlson 2000; Eschmeyer 2014). *Centropyge heraldi* is distributed in the western and central Pacific Ocean, while *C. woodheadi*, under its current definition, occurs in the southern half of the



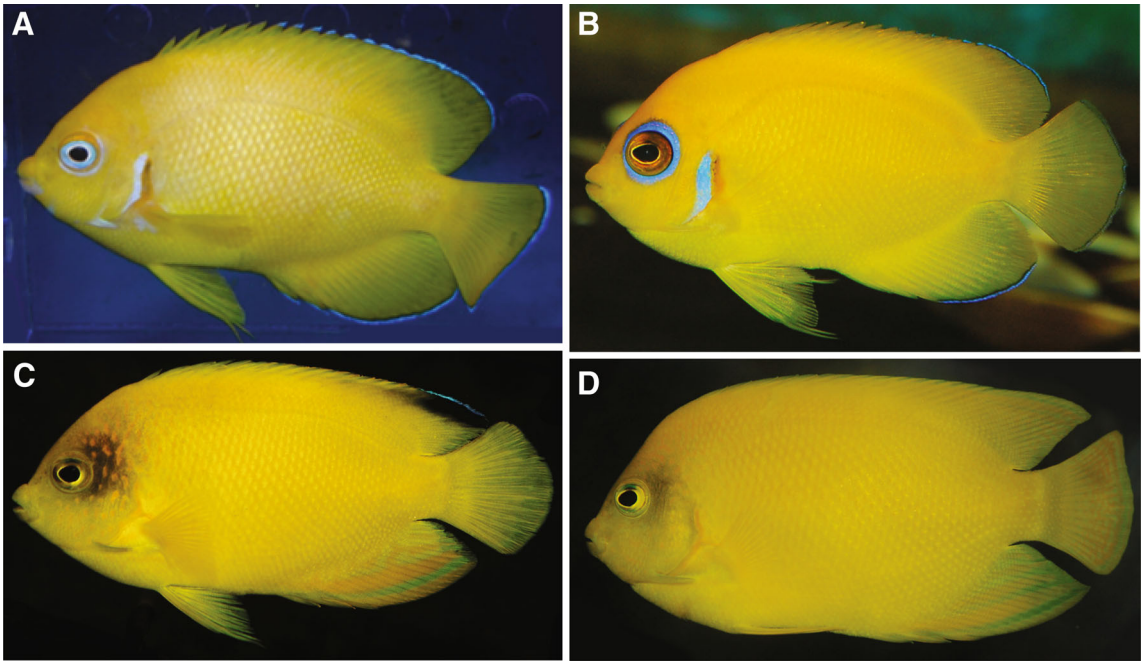


Fig. 2 Specimens representative of four yellow pygmy angelfish species, *Centropyge* spp. **a** *Centropyge cocosensis* sp. nov., no. NMMB-P19875-1 (paratype) from the Cocos Islands. **b** *Centropyge flavissima*, no. NMMB-P20403-1 from Fiji. **c** *Centropyge woodheadi*, no. NMMB-P20401-2 from the Great Barrier Reef. **d** *Centropyge heraldi*, no. NMMB-P20400 from Taiwan

tropical western Pacific Ocean (Fig. 1b) (Froese and Pauly 2012). *Centropyge woodheadi* has a black blotch of variable size at the rear end of the dorsal fin, absent in *C. heraldi* (Fig. 2c, d). Whether this colour variant deserves species status remains unclear. In the absence of apparent meristic or morphometric differences with *C. heraldi*, the observation of aquarium-kept *C. woodheadi* specimens losing their black pigmentation on the dorsal fin has been interpreted as a confirmation that the differences in colour patterns were merely phenotypic (Randall and Carlson 2000). However, R.H. Kuiter (pers. comm., December 2013) pointed out that the reverse phenomenon, i.e. full-yellow individuals acquiring a black blotch, has not yet been observed in *C. heraldi* from the northern Pacific Ocean. Also, substantial nucleotide divergence at the *CO1* locus between typical *C. heraldi* from the Philippines and Sri Lanka and the *woodheadi* form of *C. heraldi* collected from Fiji and Tonga has been reported and viewed as supporting the resurrection of *C. woodheadi* as a valid species (Steinke et al. 2009).

The present study focuses on the genetic relationships of *C. flavissima* from the Indian and Pacific Oceans. It also compares the *woodheadi* form to the typical form of *C. heraldi*, using a range of samples from all over the distribution of the two forms. The results, together with colour pattern differences and considerations on distribution patterns, lead us to challenge the current taxonomy of both *C. flavissima* and *C. heraldi*.

Materials and methods

Sampling

Specimens of the lemonpeel pygmy angelfish (Fig. 2a, b) were obtained from the Cocos (Keeling) Islands ($N = 6$),

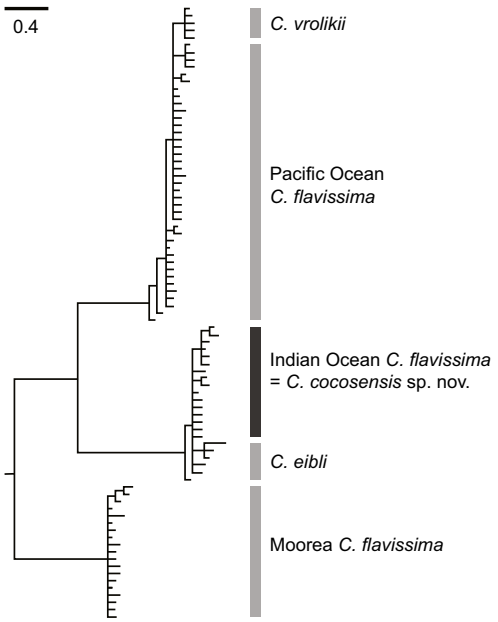


Fig. 3 Phylogenetic tree of mitochondrial DNA cytochrome *b* haplotypes (594 base pairs) for *C. flavissima*, *C. eibli* and *C. vrolikii*, based on Bayesian inference. Scale bar: 0.4 substitution/site. Modified from DiBattista et al. (2012)

New Caledonia ($N=7$), Vanuatu ($N=5$), Fiji ($N=5$), Kiribati ($N=5$) and French Polynesia, including the Australes, Gambier and Marquesas archipelagoes (total $N=13$) (Table 1). In addition, three presumed *C. flavissima* × *C. vrolikii* hybrids were collected, one from New Caledonia and two from Vanuatu (Table 1). The specimens from Cocos, Vanuatu, Fiji and Kiribati were purchased from a trusted commercial importer of aquarium fish in Taiwan. Specimens from New Caledonia and French Polynesia were collected by rotenone poisoning. We also obtained specimens of *C. eibli* from Bali ($N=3$) and *C. vrolikii* from Cebu ($N=5$), from aquarium fish traders.

New specimens of the yellow pygmy angelfish included *C. heraldi* from Taiwan ($N=1$), the Philippines ($N=3$), Bali ($N=2$) and an undisclosed sampling site in Indonesia ($N=1$); *C. heraldi* (*heraldi* form) from the Great Barrier Reef ($N=5$) and the Gambier archipelago ($N=2$); and *C. heraldi* (*woodheadi* form) from the Great Barrier Reef ($N=5$), Vanuatu ($N=6$) and Fiji ($N=2$). All new yellow pygmy angelfish specimens were either obtained from trusted aquarium fish traders (Taiwan, Philippines, Indonesia, Great Barrier Reef, Vanuatu, Fiji) or collected by rotenone poisoning during a dedicated barcoding campaign (Gambier archipelago). The distribution ranges for the nominal form and the *woodheadi* form of *C. heraldi* species are shown in Fig. 1.

A total of 106 wild-caught *Centropyge* spp. specimens was analysed for this study (Table 1). Of these, 74 came from aquarium retailers; this includes 17 specimens from Sri Lanka, Vietnam, Philippines and Tonga barcoded by Steinke et al. (2009). The remainder ($N=32$) were collected during dedicated barcoding campaigns.

Genetic analyses

A small piece of muscle tissue was excised from the dorsal part of the body and preserved in 95 % ethanol prior to DNA extraction. Genomic DNA was extracted using the DNA purification kit of Bioman (Taipei), preserved in Tris-EDTA buffer and then quantified and diluted to 1 ng/μl prior to polymerase chain reaction (PCR). Two mitochondrial genes (*16S* and *CO1*) and an intron of a nuclear oncogene (*ETS-2*) were used as genetic markers. PCR followed protocols detailed by Shen et al. (2012). Sequences were analysed in an automated ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) at the Taiwan Normal University Sequencing Facility (Taipei).

Nucleotide sequences, including those obtained in the present study and homologous sequences retrieved from the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/), were aligned using BioEdit (Hall 1999). Individual nucleotide sequences of the *16S* rDNA ($N=88$ ingroup sequences), the *CO1* gene ($N=101$) and the *ETS-2* intron ($N=63$) (Table 1) were aligned over 597 bp, 647 bp and 439 bp, respectively. For the *16S* rDNA fragment, the first nucleotide of the alignment started at the nucleotide site homologous to nucleotide site no. 1099 of the *16S* rDNA of flame angelfish *C. loriculus* (GenBank no. NC_009872); for the *CO1* fragment, the alignment started at homologous site no. 52 of the *CO1* gene in the same species. The numbering of nucleotides of the *ETS-2* intron fragment was the same as on the homologous fragment in *C. nox* previously deposited in GenBank (JQ904576). Prior to phylogenetic analysis, the matrix of *ETS-2* genotypes was transformed into a matrix of haplotypes ($N=126$) using Phase v.2.1 (Stephens et al. 2001) implemented in DnaSP v.5.10 (Librado and Rozas 2009).

Data analysis

Phylogenetic trees were reconstructed using the maximum likelihood (ML) and neighbour-joining (NJ) algorithm implemented in MEGA6 (Tamura et al. 2013). M. Kimura's 2-parameter model with gamma-distributed rate variation among sites (K2 + G) was selected as the best model of nucleotide substitution according to the Bayesian information criterion (MEGA6) for each of the *16S* rDNA, the *CO1* gene and the *ETS-2* intron marker. All sequences obtained in this study were deposited in GenBank (see Table 1). Homologous sequences of sixbar angelfish *Pomacanthus sexstriatus* and yellowface angelfish *P. xanthometopon* (GenBank nos. KJ542547, KJ542548, KJ551856–KJ551858, KJ624708, KJ624709, KJ624691–KJ624693) were used as outgroups.

Hierarchical analysis of molecular variance (AMOVA: Excoffier et al. 1992) on each of the *16S*, *CO1* and *ETS-2* sequence datasets for each of *C. flavissima* and *C. heraldi* was done under Arlequin v.3.5 (Excoffier and Lischer 2010) to examine the partitioning of the total variance among regional groups of samples. Two groups of populations, Indian vs. Pacific, were considered in *C. flavissima* based on patterns of geographic distribution (Fig. 1b). Two groups, northern Pacific vs. southern Pacific, were similarly considered in *C. heraldi* (Fig. 1c). Data files were prepared under DnaSP. The best substitution model according to the Bayesian information criterion was determined for each dataset with MEGA6. Significance of the Φ-statistics and associated variance components was tested by 1000 random permutations (Arlequin).

Measurements on type specimens

Morphometric measurements and meristic counts were done on the type material of the Indian Ocean form of the lemonpeel pygmy angelfish, following Randall and Rocha (2009). Standard length, body depth, caudal peduncle length, caudal peduncle depth, pre-dorsal length, pre-anal length, pre-pelvic length, lengths of the fin spines and rays, and caudal fin length were taken from X-ray images of the specimens, using

Table 1 Specimens of pygmy angelfishes (*Centropyge* spp.) of the *C. flavissima* and *C. heraldi* species complexes analysed for nucleotide sequence variation at the *16S*, *CO1* and *ETS-2* loci, with sampling details, specimen numbers and GenBank accession numbers

Species, sampling location	Sampling date	*N*	Individual no.	Voucher	Photograph	Locus		
						16S	*CO1*	*ETS-2*
C. cocosensis sp. nov.								
Cocos (Keeling) Is.	Aug. 2010	1	104	NMMB-P19870 (paratype)	–	KJ551861	KJ534314	KJ624654
Cocos (Keeling) Is.	Oct. 2011	1	173	NMMB-P19875-1 (paratype)	Fig. 2a	KJ551862	KJ534315	KJ624655
Cocos (Keeling) Is.	Oct. 2011	1	174	NMMB-P19875-2 (holotype)	–	KJ551863	KJ534316	KJ624656
Cocos (Keeling) Is.	Oct. 2011	1	318	USNM 410766 (paratype)	–	KJ643461	KJ534317	KJ624659
Cocos (Keeling) Is.	Feb. 2012	1	359	NMMB-P20395	–	KJ551865	KJ534318	KJ624658
Cocos (Keeling) Is.	Dec. 2013	1	P4	NMMB-P20399	–	KJ551864	KJ534319	KJ624657
C. eibli								
Vietnam	Oct. 2006	2	–	HLC-15215, -15216	BOLD	–	FJ5829560, FJ582959	–
Bali	Jun. 2010	1	48	NMMB-P19743	–	KJ551867	KJ534321	–
Bali	Sep. 2010	1	113	NMMB-P19745	–	KJ551866	KJ534320	KJ624660
Bali	Dec. 2013	1	P17	NMMB-P20397	–	KJ551868	KJ534322	KJ624661
C. flavissima								
New Caledonia	Dec. 2010	7	45–51	MNHN IC-2010-1332 to -1334	BOLD	–	KJ542555–KJ542561	KJ624695–KJ624697
Vanuatu	May 2010	5	73–77	NMMB-P19868-1 to -5	–	KJ551870, KJ551873– KJ551876	KJ534323–KJ534327	KJ624662–KJ624665
Fiji	Dec. 2013	1	P5	NMMB-P20403-1	Fig. 2b	KJ551878	KJ534351	KJ624688
Fiji	Dec. 2013	4	P6, P11–P13	NMMB-P20403-2 to -5	–	KJ551880, KJ551882, KJ551886, KJ551888	KJ534352–KJ534354	KJ624689–KJ624690
Nuku'alofa, Tonga	Sep. 2005	1	–	HLC-10882	BOLD	–	FJ582964	–
Rapa, Australes Is.	Nov. 2002	6	18, 20–24	–	–	KJ643441, KJ643443– KJ643447	KJ624645–KJ624650	KJ624702–KJ624705
Moorea, Society Is.	Mar. 2006	4	556, 557, 558, 559	MNHN 2008-720, 2008-220, 2008-219, 2008-719	BOLD	–	JQ431556–JQ431559	–
Moorea, Society Is.	Fev. 2009	1	941	FLMOO 446	BOLD	–	KJ967941	–
Line Is., Kiribati	Jan. 2014	5	P19–P23	NMMB-P20404-1 to -5	–	KJ551881, KJ551884, KJ551885, KJ551887, KJ551889	KJ534328–KJ534332	KJ624666–KJ624669
Gambier	Sep.–Oct. 2010	2	F1, F2	GAM-039, GAM-786	–	KJ643457, KJ643458	KJ624651, KJ624652	KJ624706

Table 1 (continued)

Species, sampling location	Sampling date	*N*	Individual no.	Voucher	Photograph	Locus		
						16S	*CO1*	*ETS-2*
Marquesas Islands	Oct. 2011	7	28–31, 35–37	–	–	KJ643450–KJ643456	KJ624640–KJ624644	KJ624698–KJ624701
C. flavissima × *C. vrolikii*[a]								
New Caledonia	Dec. 2010	1	NC	MNHN IC-2010-1535	–	KM453706	KM453711	KM453716
Vanuatu	Dec. 2009	1	54	NMMB-P20394	–	KM453707	KM453712	KM453718
Vanuatu	Jul. 2012	1	336	NMMB-P19867	–	KM453708	KM453713	KM453717
C. heraldi[b]								
Sri Lanka	Oct. 2006	1	966	HLC-15168	–	–	FJ582966	–
Taiwan	Dec. 2013	1	P3	NMMB-P20400	Fig. 2d	KJ551909	KJ534345	KJ624682
Philippines	–	4	968, 969, 972, 973	HLC-11144, -11143, -10794, -10793	BOLD	–	FJ582968, FJ582969, FJ582972, FJ582973	–
Philippines	Jul. 2010	1	116	NMMB-P19872	–	KJ551906	KJ534343	KJ624679
Philippines	Aug. 2012	1	156	NMMB-P19876-1	–	KJ551907	KJ534344	KJ624680
Philippines	Aug. 2012	1	324	NMMB-P19876-2		KJ551908	KM434228	KJ624681
Bali	Feb. 2014	2	HB1, HB2	NMMB-P20756-1, -2		KM453709, KM453710	KM453714, KM453715	KM453719, KM453720
Indonesia	Apr. 2012	1	408	NMMB-P20759		KJ551910	KM434227	KM434230
C. vrolikii								
Cebu	Feb. 2010	3	Cebu3, 4, 5	NMMB-P20754-1 to -3	–	KJ551871, KJ551872, KJ551877	KJ542550–KJ542552	KJ764882
Cebu	Dec. 2013	2	P15, P16	NMMB-P20396, -20398	–	KJ551879, KJ551883	KJ542553, KJ542554	KJ624694, KJ764883
Cebu	Dec. 2009	1	55	NMMB-P20758	–	KJ551869	KJ542549	KJ764884
Philippines	Sep.–Dec. 2005	4	–	HLC-11065, -11638, -11639, -11715	BOLD	–	FJ583001, FJ582998, FJ582997, FJ583002	–
Nuku'alofa, Tonga	Sep. 2005	2	–	HLC-11078, -11079	BOLD	–	FJ582996, FJ582999	–
C. woodheadi								
Great Barrier Reef[c]	Feb. 2011	2	144, 146	NMMB-P19873-1, -2	–	KJ551890, KJ551891	KJ534333, KJ534334	KJ624670, KJ624671
Great Barrier Reef[c]	Dec. 2013	2	P1, P14	NMMB-P20401-1, -3	–	KJ551903, KJ551911	KJ534335, KJ534337	KJ624672, KJ624674
Great Barrier Reef[c]	Dec. 2013	1	P7	NMMB-P20401-2	Fig. 2c	KJ551898	KJ534336	KJ624673
Great Barrier Reef[d]	Dec 2013	5	P2, P8–P10, P18	NMMB-P20402-1 to -5	–	KJ551899, KJ551901, KJ551892, KJ551905	KJ534338–KJ534342	KJ624675–KJ624678

Table 1 (continued)

Species, sampling location	Sampling date	*N*	Individual no.	Voucher	Photograph	Locus		
						16S	*CO1*	*ETS-2*
Vanuatu[c]	Feb. 2011	2	303, 304	NMMB-P19874-1, -2	–	KJ551892, KJ551895	KJ534347, KJ534348	KJ624685, KJ624686
Vanuatu[c]	Apr. 2011	2	151, 152	NMMB-P19877-1, -2	–	KJ551893, KJ551894	KJ534345, KJ534346	KJ624683, KJ624684
Vanuatu[c]	Feb. 2013	1	I003	NMMB-P19880	–	KJ551904	KJ534349	KJ624687
Vanuatu[c]	Feb. 2014	1	387	NMMB-P20760	–	KM434225	KM434226	KM434229
Fiji[c]	May 2013	2	I001, I002	NMMB-P19881-1, -2	–	KJ551896, KJ551897	KJ534350	KJ764880, KJ764881
Nuku'alofa, Tonga[c]	Sep. 2005	3	965, 967, 970	HLC-15119, -12131, -11027	BOLD	–	FJ582965, FJ582967, FJ582970	–
Moorea, Society Is.[d]	Mar. 2006	2	560, 561	MBIO1259.4, MBIO974.4	BOLD	–	JQ431560, JQ431561	–
Gambier[d]	Sep.–Oct. 2010	2	H1, H2	GAM-085, GAM-416	–	KJ643459, KJ643460	KJ624653	KJ624707

BOLD Barcode of Life Database (http://www.barcodinglife.com/); *MNHN* Muséum National d'Histoire Naturelle, Paris; *NMMB* National Museum of Marine Biology and Aquarium, Pingtung; *USNM* United States National Museum, Washington DC; *N* sample size

[a] Presumed hybrids

[b] As originally defined (Schultz et al. 1953); the full-yellow *C. heraldi* individuals sampled from the western and central South Pacific were eventually determined as *C. woodheadi* based on molecular markers (present study)

[c] *woodheadi* form as originally defined (Kuiter 1998), i.e. possessing a black blotch at the rear end of the dorsal fin

[d] *heraldi* form, i.e. without black blotch on the dorsal fin, determined as *C. woodheadi* from molecular markers (present study)

the X-ray imager (XL-100, Laiko Co., Tokyo) of the National Museum of Marine Biology and Aquarium in Pingtung (Shen et al. 2012). The other measurements (Supplementary Table S1) were taken directly on the fish using electronic calipers and were rounded to the nearest 0.1 mm. Dorsal, anal and caudal fin spines and rays were counted from X-ray images (Shen et al. 2012).

Results

Hierarchical analysis of molecular variance

The components of molecular variance according to the hierarchical level of grouping are summarised in Table 2. Here, we are interested in the percentage of genetic variation that can be assigned to differences between groups of populations defined geographically. Fisher's combined probability test (Sokal and Rohlf 1969) on the exact probabilities of the Φ_{CT} values allowed the rejection of the null hypothesis ($\Phi_{CT}=0$; no genetic differentiation between groups) in both *C. flavissima* ($P=0.043$) and *C. heraldi* ($P<0.001$). The Φ_{CT} values in both cases ($\Phi_{CT}=0.438$–0.484 in *C. flavissima*; $\Phi_{CT}=0.752$–0.900 in *C. heraldi*) corresponded to high levels of genetic differentiation, indicative of separate species (see Fauvelot and Borsa 2011; Borsa et al. 2012; Borsa et al. 2016 for comparisons of estimates of genetic differentiation across shorefish species, where values >0.400 are observed between species in species complexes, not among populations within a species).

Mitochondrial phylogeny of four yellow Indo-Pacific *Centropyge* spp.

In both *16S* rDNA and *CO1* gene phylogenies (Fig. 4a, b), *C. flavissima* mitochondrial haplotypes from Rapa and the Gambier archipelago formed a subclade sister to all the other *C. flavissima*. This subclade also included haplotypes from Moorea, as seen from the *CO1* tree (Fig. 4b). Within the main *C. flavissima* subclade, the Marquesas haplotypes represented a distinct lineage. Haplotypes of the Cocos (Keeling) Islands form of the lemonpeel pygmy angelfish belonged to a

Table 2 Analysis of molecular variance (AMOVA; Excoffier et al. 1992) among populations of the *Centropyge flavissima* and *C. heraldi* species complexes based on the geographic partition presented in Fig. 1a (*C. flavissima* vs. *C. cocosensis* sp. nov.) and Fig. 1b (*C. heraldi* vs. *C. woodheadi*); the Sri Lanka and Bali *C. heraldi* samples were arbitrarily grouped with northern tropical Pacific *C. heraldi*

Species complex, source of variation, marker	d.f.	% variation	*F*-statistic	*P*-value
C. flavissima				
Among groups				
16S	1	44.1	$\Phi_{CT}=0.440$	0.114
CO1	1	43.8	$\Phi_{CT}=0.438$	0.116
ETS-2	1	48.4	$\Phi_{CT}=0.484$	0.113
Among populations within groups				
16S	6	51.1	$\Phi_{SC}=0.914$	<0.001
CO1	8	54.6	$\Phi_{SC}=0.971$	<0.001
ETS-2	6	−1.1	$\Phi_{SC}=-0.021$	0.024
Among populations relative to total				
16S	41	4.8	$\Phi_{ST}=0.952$	<0.001
CO1	34	1.6	$\Phi_{ST}=0.984$	<0.001
ETS-2	50	52.7	$\Phi_{ST}=0.473$	<0.001
C. heraldi				
Among groups				
16S	1	85.4	$\Phi_{CT}=0.854$	0.030
CO1	1	90.0	$\Phi_{CT}=0.900$	0.002
ETS-2	1	20.5	$\Phi_{CT}=0.752$	0.029
Among populations within groups				
16S	5	−1.6	$\Phi_{SC}=-0.111$	0.800
CO1	8	7.6	$\Phi_{SC}=-0.005$	0.568
ETS-2	5	1.0	$\Phi_{SC}=-0.077$	0.833
Among populations relative to total				
16S	20	16.2	$\Phi_{ST}=0.838$	<0.001
CO1	25	24.0	$\Phi_{ST}=0.899$	<0.001
ETS-2	43	15.4	$\Phi_{ST}=0.733$	<0.001

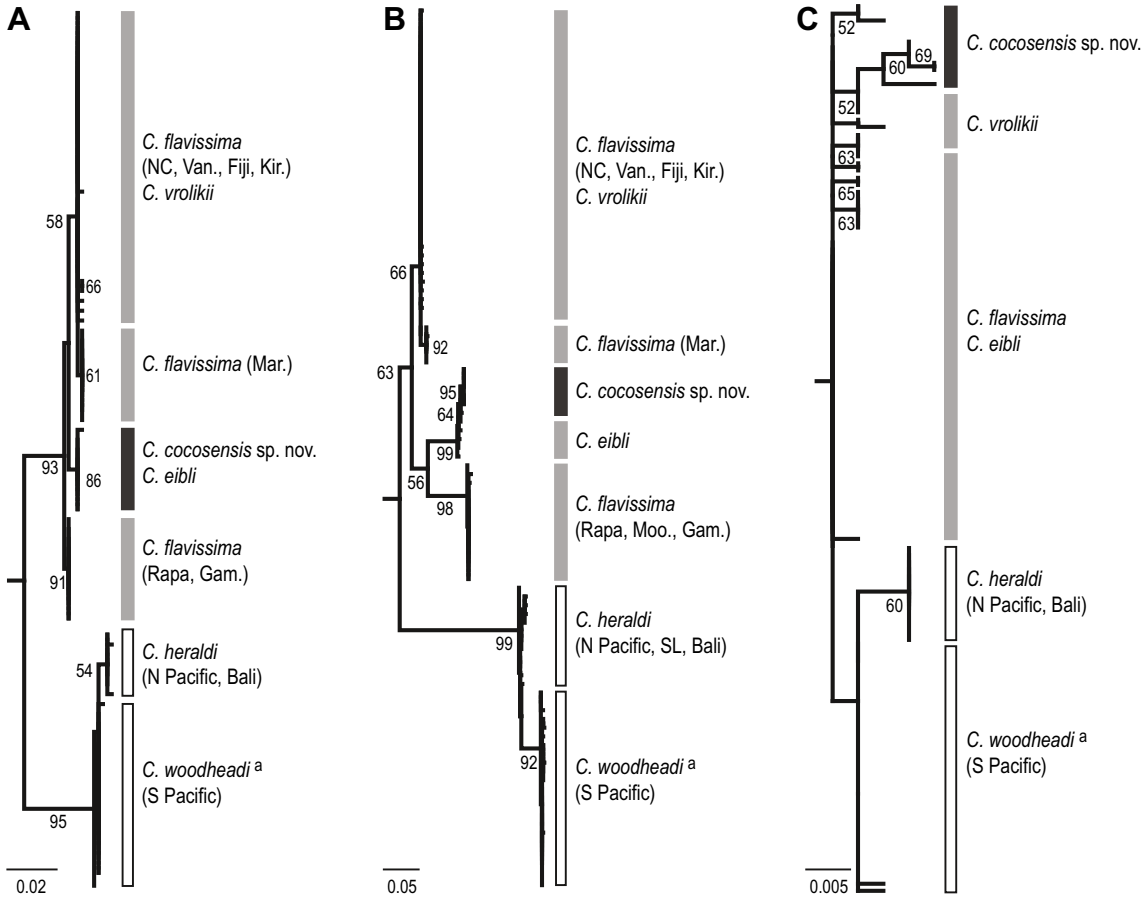


Fig. 4 Phylogeny of yellow pygmy angelfishes, *Centropyge* spp.: maximum likelihood (ML) trees of nucleotide sequences (list in Table 1) reconstructed using MEGA6 (Tamura et al. 2013). The numbers at nodes are bootstrap scores in % (1000 random re-sampling runs; MEGA6); bootstrap scores below 50 % are not shown. *Gam.* Gambier islands; *Kir.* Kiribati; *Mar.* Marquesas islands; *Moo.* Moorea; *NC* New Caledonia; *Phi.* Philippines; *SL* Sri Lanka; *Van.* Vanuatu. [a] Haplotypes designating *C. woodheadi* under its new definition (Table 1). **a** ML tree (based on the K2 + G model) of 88 *16S* rDNA sequences aligned over 597 bp, rooted by homologous sequences of *Pomacanthus xanthometopon* (GenBank KJ551856–KJ551858). **b** ML tree (K2 + G model) of 101 *CO1* gene sequences aligned over 647 bp, rooted by homologous sequences of *P. sexstriatus* (GenBank KJ542547, KJ542548) and *P. xanthometopon* (GenBank KJ534356–KJ534358). **c** ML tree (K2 + G model) of 126 intron *ETS-2* haplotype sequences aligned over 439 bp, rooted by homologous sequences of *P. sexstriatus* (GenBank KJ624708, KJ624709) and *P. xanthometopon* (GenBank KJ624691–KJ624693)

subclade distinct from Pacific *C. flavissima* (Fig. 4a, b). Haplotypes of the Cocos form were closest to *C. eibli*, although distinct from the latter, as visible from the *CO1* phylogeny (Fig. 4b) and from analysis of the concatenated sequence dataset (Supplementary Material, Figs. S2 and S3); *C. vrolikii* haplotypes were shared with those of western-Pacific Ocean *C. flavissima*, i.e. those sampled in New Caledonia, Vanuatu, Fiji and Kiribati.

Haplotypes of the *woodheadi* form of *C. heraldi* clustered with all other southern-Pacific Ocean *C. heraldi*, as a subclade sister to northern-Pacific Ocean and Indian Ocean *C. heraldi* (Fig. 4a, b; Supplementary Material, Figs. S2 and S3).

Nuclear phylogeny

The Cocos (Keeling) Islands and the Pacific forms of lemonpeel pygmy angelfish mostly differed by a single G/C transversion along the 438-bp-long fragment of the *ETS-2* intron. Eight out of 12 *ETS-2* haplotypes sampled from Cocos possessed C (4/12 with G) at nucleotide site 180 of the fragment, while all 23 *C. flavissima* individuals sampled from the Pacific Ocean were homozygous for nucleotide G. The presence of C at nucleotide site no. 180 was systematically associated with T at sites nos. 219 and 225, while G at site no. 180 was mostly associated with C at sites nos. 219 and 225, thus defining two major alleles in lemonpeel pygmy angelfish, hereafter coined *CTT* and *GCC*. One individual from the Cocos population of the lemonpeel pygmy angelfish was heterozygous for the possibly recombinant *CCC* allele and another individual was heterozygous for the possibly recombinant *CTC* allele. Allele *CTT* was dominant in frequency in the Cocos population and absent from Pacific *C. flavissima* and *C. eibli* samples. Two alleles, *GCC* and *GTC*, were recorded in *C. vrolikii*. In the nuclear phylogeny (Fig. 4c),

haplotypes sampled in the Cocos form of lemonpeel pygmy angelfish generally clustered separately from the other *C. flavissima* haplotypes. The latter were shared or mixed with *C. eibli* and *C. vrolikii* haplotypes and with 2/12 haplotypes from the Cocos lemonpeel pygmy angelfish. In the NJ tree (not shown), all Cocos lemonpeel haplotypes clustered as a single lineage.

All *ETS-2* intron haplotypes of the *woodheadi* form of *C. heraldi* clustered together with all other southern-Pacific *C. heraldi*, separately from northern-Pacific *C. heraldi* (Fig. 4c). Northern-Pacific *C. heraldi* intron haplotypes were characterised by T at nucleotide site no. 90 and C at site no. 187, whereas the *woodheadi* form and southern-Pacific *C. heraldi* possessed A and T, respectively, at these sites.

Morphology

For the sake of formal taxonomic description, morphological measurements for four lemonpeel pygmy angelfish individuals from the Cocos (Keeling) Islands, including the holotype and the three paratypes, are presented in Supplementary Table S1. The only salient feature that distinguished Indian Ocean *C. flavissima* from its Pacific counterpart was the colour of the iris (Fig. 2), as noted previously (e.g. DiBattista et al. 2012: their Fig. 2).

Discussion

Species in the *C. flavissima* species complex (*C. flavissima*, *C. eibli* and *C. vrolikii*) are represented by deep mitochondrial lineages that characterise geographic region rather than species designation as based on colour patterns. It has been proposed that ancient mitochondrial divergences between species within this complex have been erased by subsequent introgression (DiBattista et al. 2012; Gaither et al. 2014). In the present study, the phylogenetic analyses based on both mitochondrial and nuclear genes showed that the Indian Ocean form of the lemonpeel pygmy angelfish is genetically different not only from its counterpart *C. flavissima* from the western and central Pacific, but also from the sympatric *C. eibli* and from *C. vrolikii*. This difference in haplotype composition indicates that, despite inferred past introgression between the Cocos form of the lemonpeel pygmy angelfish and *C. eibli*, gene flow between the two species has since been low or even, perhaps, absent.

Species are separately evolving segments of the global genealogical network of living organisms (de Queiroz 2007; Barberousse and Samadi 2010). To hypothesise the existence of distinct species, researchers practically have access to empirical lines of evidence which include reproductive isolation from other species, the possession of fixed character state differences and monophyly (de Queiroz 2007). Hampered gene flow in a situation of sympatry implies some degree of reproductive isolation (Mayr 1942), an expected property of separate species. Reproductive isolation, separation of mitochondrial haplotypes and partial separation of nuclear haplotypes thus provide evidence that the Cocos form of the lemonpeel pygmy angelfish is an entity genetically independent from the sympatric *C. eibli*. The occurrence of *C. cocosensis* sp. nov. × *C. eibli* hybrids at Cocos (Keeling) Islands (Hobbs and Allen 2014) does not contradict our conclusion. When confronted with an apparent deficit of shared haplotypes between the two species (DiBattista et al. 2012), this observation actually indicates that hybrids have lower survival or fertility than individuals from the parental species. Also, under the hypothesis of a single species, one would expect to observe a gamut of hybrid colour morphs against only a small proportion of pure morphs, something that has not yet been reported (DiBattista et al. 2012; Hobbs and Allen 2014).

The Indian Ocean form of the lemonpeel pygmy angelfish has a unique blue iris that is absent in *C. flavissima* and it has a much less pronounced blue eye ring than that of *C. flavissima*. The blue iris is also characteristic of the local lemonpeel pygmy angelfish population from Christmas Island (Allen and Erdmann 2012; Froese and Pauly 2012). Colour differences are maintained despite gene flow between *C. cocosensis* and *C. eibli* in the Indian Ocean, as they are between *C. flavissima* and *C. vrolikii* in the Pacific Ocean. The loci involved in colour differentiation are impermeable to gene flow, revealing a reproductive barrier—at least a partial one—which is the biological signature of separate species. Here, we use the conspicuous iris colour as the character that diagnoses the geographically and genetically partly isolated Indian Ocean lemonpeel pygmy angelfish as a separate species. The evolutionary mechanisms that have led to the colour differences between the Indian and Pacific forms of the lemonpeel pygmy angelfish may include genetic drift, enhanced by the presumably small effective population size of the Indian Ocean form, and sexual selection. Sexual selection may also be invoked to explain the dominance of the pure parental morphs over hybrids. Further research is warranted to test these hypotheses.

Six nominal species are currently considered synonyms of *C. flavissima* (Eschmeyer 2014). These are *Holacanthus cyanotis* Günther, 1860, *H. luteolus* Cuvier and Valenciennes, 1831, *H. monophthalmus* Kner, 1867, *H. ocularis* Peters, 1868, *H. sphynx* DeVis, 1864 and *H. uniocellatus* Borodin, 1932. The type localities of the foregoing species are in the Pacific Ocean, except for *H. ocularis*, which is from the '*Südsee*,' a term that designates both the tropical Indian and Pacific Oceans. However, *H. ocularis* is described as '*ganz gelb*' and has "*um jedes Auge*

ein blauer schwarz eingefasster Ring und auf dem hinteren Rande des Operculums eine senkrechte, hellblaue schwarz eingefasste Binde" (Peters 1868). Thus, the description of W.C.H. Peters' *H. ocularis* colour patterns is typical of Pacific *C. flavissima*, leaving the Indian Ocean form of the lemonpeel pygmy angelfish still undescribed, hence clearing the way for its description as a new species (International Commission on Zoological Nomenclature 1999).

Centropyge heraldi, under its current definition, is distributed throughout the central and western Pacific, while the blackfin or *woodheadi* form solely occurs in southern tropical western and central Pacific. The two forms co-occur in the north-western part of the Coral Sea (Debelius et al. 2003), in Fiji and in the Society Islands (Randall and Carlson 2000), but not in Samoa, where the blackfin form is the sole present (Randall and Carlson 2000). The present study indicates that *C. heraldi* actually consists of two genetically distinct anti-tropical populations, while no genetic character allowed the distinction of the *woodheadi* form from sympatric, southern-Pacific *C. heraldi*. Reciprocal monophyly characterised the southern Pacific vs. northern hemisphere populations. Therefore, the southern Pacific and the northern hemisphere populations of *C. heraldi* should be considered as separate evolutionary units. Epithet *woodheadi*, which is available, should be resurrected for the southern Pacific population.

Taxonomy

Centropyge cocosensis, new species http://zoobank.org/4B6BB6D8-9B7D-4128-B7FD-F8AA1D04693E

Previous references. Centropyge flavissima (Allen et al. 1998, 2003; Allen and Erdmann 2012; DiBattista et al. 2012; Gaither et al. 2014; Hobbs and Allen 2014).

Vouchers The type material of *C. cocosensis* sp. nov. was deposited at the National Museum of Marine Biology and Aquarium (NMMB) in Pingtung, Taiwan and at the United States National Museum (USNM) in Washington DC, USA, under nos. NMMB-P19870 (paratype), NMMB-P19875-1 (paratype), NMMB-P19875-2 (holotype) and USNM 410766 (paratype). Two additional vouchers were deposited at NMMB (Table 1). All the foregoing specimens were collected from the Cocos (Keeling) Islands.

Description Meristic counts and morphological measurements on holotype and paratypes are presented in Supplementary Table S2. The morphological description and the *16S*, *CO1* and *ETS-2* nucleotide sequences of the holotype are also provided in the Supplementary Material.

Comparison with closely related species *Centropyge cocosensis* sp. nov. is closely related to the group of pygmy angelfishes which includes *C. eibli*, *C. flavissima* and *C. vrolikii*. The four species cluster as a single clade in the multiple-locus phylogeny of the group (Gaither et al. 2014). Both *C. cocosensis* sp. nov. and *C. flavissima* possess a blue eye ring, but the eye ring is paler, narrower and fainter in the former.

Diagnosis Colour of live specimens lemonpeel yellow with blue margined gill operculum, spine and fins; conspicuous blue iris (iris is golden yellow in *C. flavissima*) and faint blue eye ring surrounding the eye (Fig. 2a); colour in alcohol uniformly yellowish with dark eye and dark posterior dorsal, anal and caudal fin margins. *Centropyge cocosensis* sp. nov. can be separated from *C. flavissima* and the two other species of the *C. flavissima* species complex by nucleotide quasi-synapomorphies at the *CO1* locus. These include G at both nucleotide sites 247 and 366 of the gene.

Distribution Known from the Cocos (Keeling) Islands (present study) and from the nearby Christmas Island in the East Indian Ocean (Allen et al. 1998; DiBattista et al. 2012; Hobbs and Allen 2014).

Etymology Epithet *cocosensis* is the Latin derivation of Cocos, from Cocos (Keeling) Islands, the type locality of the new species. As the vernacular name, we propose that the new species be called Cocos pygmy angelfish.

Remarks In their original description of *C. flavissima* ("*L'Holacanthe tout-jaune*") from the drawing of a specimen from Ulea, Cuvier and Valenciennes (1831) did not mention the blue eye ring and other blue ornaments. It is likely that the blue colour of the specimen had faded after death. The type locality (Ulea) is in the Central Pacific, hence there is no ambiguity that the two authors referred to the Pacific form of *C. flavissima*.

Centropyge woodheadi Kuiter, 1998, resurrected species

Previous references *Centropyge woodheadi* (Kuiter 1998; Seeto and Baldwin 2010); *C. heraldi* (non Woods and Schultz, 1953) (Randall 1997, 2005; Laboute and Grandperrin 2000; Randall and Carlson 2000; Allen et al. 2003; Steinke et al. 2009).

Re-description *Centropyge woodheadi* is here re-described from nucleotide sequences at the *16S* rDNA, *CO1* gene and *ETS-2* intron. Based on the individual sequences listed in Table 1, *C. woodheadi* possesses (C, T) at nucleotide site positions nos. (1378, 1479) of the *16S* rDNA; (A, G, A, G, C, C, C, G, T, A, G, G, C, T) at nucleotide site positions nos. (147, 168, 255, 342, 444, 453, 495, 540, 621 630, 645, 678, 687, 690) of the *CO1* gene; and (A, T) at nucleotide site positions nos. (90, 187) of the *ETS-2* fragment. The foregoing

molecular characters distinguish it from *C. heraldi* under its present new definition (see the next sub-section).

Distribution Western and central South Pacific Ocean, from the Great Barrier Reef to the Gambier archipelago (Table 1; Fig. 1c).

Suggested vernacular name Keeping the vernacular name coined previously for *C. woodheadi* (the "blackfin pygmy angelfish"; Kuiter 1998) could be misleading. This fish is called *paraharaha* in Tahiti (Froese and Pauly 2012). We suggest that this name be used to distinguish *C. woodheadi* under its present, new definition, from the yellow pygmy angelfish, *C. heraldi*.

Centropyge heraldi Woods and Schultz, 1953

Re-description *Centropyge heraldi* is here re-described from nucleotide sequences at the *16S* rDNA, *CO1* gene and *ETS-2* intron. Based on the sample of individual sequences listed in Table 1, *C. heraldi* possesses (T, C) at nucleotide site positions nos. (1378, 1479) of the *16S* rDNA; (C, A, G, A, T, T, T, A, A, C, A, A, T, C) at nucleotide site positions nos. (147, 168, 255, 342, 444, 453, 495, 540, 621 630, 645, 678, 687, 690) of the *CO1* gene; and (T, C) at nucleotide site positions nos. (90, 187) of the *ETS-2* fragment. The foregoing molecular characters distinguish it from *C. woodheadi* under its present new definition.

Distribution In the Indian Ocean, *C. heraldi* is known from Sri Lanka (Steinke et al. 2009) and Bali (present study), whereas in the Pacific Ocean, it has a wide distribution, from the Indo-Malay archipelago to the Marshall Islands (Table 1; Fig. 1c).

Notice

The present article in portable document (.pdf) format is a published work in the sense of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 2012) or Code and, hence, the new names contained herein are effectively published under the Code. This published work and the nomenclatural acts it contains have been registered in ZooBank (http://zoobank.org/), the online registration system for the International Commission on Zoological Nomenclature. The ZooBank life science identifier (LSID) for this publication is 4B6BB6D8-9B7D-4128-B7FD-F8AA1D04693E. The online version of this work is archived and available from the *Marine Biodiversity* and Hal-IRD repository (http://www.hal.ird.fr/) websites.

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