Original Article

MARINE BIOTECHNOLOGY

Phylogenetic Relationships of South China Sea Snappers (Genus *Lutjanus*; Family Lutjanidae) Based on Mitochondrial DNA Sequences

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Abstract

Phylogenetic relationships of intra- and interspecies were elucidated based on complete cytochrome *b* (*cyt b*) and cytochrome *c* oxidase subunit II (CO*II*) gene sequences from 12 recognized species of genus *Lutjanus* Bloch in the South China Sea (SCS). Using the combined data set of consensus *cyt b* and CO*II* gene sequences, interspecific relationships for all 12 recognized species in SCS were consistent with Allen's morphology-based identifications, with strong correlation between the molecular and morphological characteristics. Monophyly of eight species (*L. malabaricus, L. russellii, L. stellatus*, *L. bohar*, *L. johnii*, *L. sebae, L. fulvus*, and *L. fulviflamma*) was strongly supported; however, the pairs *L. vitta*/*L. ophuysenii* and *L. erythropterus*/*L. argentimaculatus* were more similar than expected We inferred that *L. malabaricus* exists in SCS, and the introgression caused by hybridization is the reason for the unexpectedly high homogeneity.

Keywords: hybridization — introgression — *Lutjanus* — mitochondrial DNA — phylogenetic relationship

Introduction

According to FishBase (http://www.fishbase.org), 67 species of genus *Lutjanus* Bloch are distributed throughout tropical and subtropical oceans worldwide. This coral reef genus is represented in the South China Sea (SCS) by approximately 20 indigenous species, which are important economically and are a significant source of food for developing countries around SCS (Russ and Alcala 1989). Dense populations of these fish are greatly decreased by overfishing, and coral is damaged by destructive fishing techniques and by removal for trade. "The State of the World Fisheries and Aquaculture" concluded that developments in world fisheries and aquaculture during recent years have continued to follow trends that were already becoming apparent at the end of the 1990s: capture fisheries production is stagnating and aquaculture output is expanding faster than any other animal-based food sector. To solve this issue, members of genus *Lutjanus* (e.g., *L. malabaricus, L. erythropterus*, *L. argentimaculatus*, *L. stellatus*, and *L. sebae*) have been cultured in Southeast Asia since 1990s because of their fast growth rate.

Compared with western Atlantic snappers (Camper et al. 1993; Gold and Richardson 1998; Gold et al. 1994, 1997; Kristmundsdo et al. 1996; Sarver et al. 1996), phylogenetic analysis of SCS snappers is poorly developed, especially because taxa are morphologically similar (Marko et al. 2004; Zhang et al. 2004) and have the ability to hybridize (Domeier and Clarke 1992; Loftus 1992; Chen et al. 2006). The economically important species *L. erythropterus* and *L. malabarius* are distributed widely in the tropical Indo-Pacific (from Gulf of Oman to Southeast Asia, northward to southern Japan and southward to northern Australia), eat the same prey species, exhibit similar maximum sizes and ages, and are often misidentified owing to morphological similarity (Allen 1985; Shen 1993; Froese and Pauly 2001). Salini et al. (2006) reported that *L. malabaricus* and *L. erythropterus* were fished commercially in northern Australian, Papua New Guinean, and Indonesian waters by commercial and recreational fishers, and investigated their genetic population structure. SCS is included in the distri-

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bution range of these species; however, *L. malabaricus* never appears in the fauna of mainland China (Institute of Zoology et al. 1962; Fishes of Fujian Province Editorial Subcommittee 1985; Cheng and Zheng 1987). The existence of *L. malabaricus* species in SCS is under dispute in China (Guo 2004; Zhu et al. 2006). In addition, *L. ophuysenii* (Bleeker) and *L. vitta* (Quoy & Gaimard) have been considered synonymous with the latter by many investigators (Yukio et al. 1993). *L. vitta* ranges widely from southern Japan (Ryukyu Is. only), southeastern China, Australia, and in most of the Indian Ocean, while *L. ophuysenii* is confined to southern Japan (excluding Ryukyu Is.), southern Korea, and the eastern and southeastern Chinese coasts, where the two species occur sympatrically. This has also been addressed by development of artificial breeding and hybridization technology for some high-value species such as *L. sebae, L. argentimaculatus, L. malabaricus*, and *L. erythropterus*.

Given the importance of different species, basic research on taxonomic status is essential for conservation of these taxa, including characterization of taxonomic diversity and development of aquaculture for SCS snappers. On the whole, there are two influential taxonomic systems of the genus *Lutjanus* in SCS. Allen (1985) dedicated decades to the taxonomic revision of genus *Lutjanus*, using body shape, body color, stripes, and spines and rays of the dorsal fin as primary distinguishing characteristics. Cheng (1987) used scales around the lateral line and opercular bones as primary taxonomic characteristics. Researchers all wish to know which taxonomic data sets most closely approximate phylogenetic relationships.

Mitochondrial studies are quite popular among aquaculture geneticists, in part owing to the mito-

Table 1. Valid names, English names, distribution, and accession numbers of all individuals

Species	*Distribution*	*Samples*	*Accession no. for COII*	*Accession no. for Cyt b*
L. russellii (Russell's snapper)	Indo-West Pacific	*L1*	DQ900714	DQ900670
		L2	DQ900715	DQ900671
		L3	DQ900716	EF376162
L. argentimaculatus (Mangrove red snapper)	Indo-West Pacific	*Z1*	DQ900723	DQ900672
		Z2	DQ900724	DQ900673
		Z3	EF025487	EF025494
L. stellatus (Star snapper)	Northwest Pacific	*X1*	DQ900701	EF376163
		X2	DQ900702	EF376164
		X3	DQ900703	DQ900662
L.bohar (Two-spot red snapper)	Indo-Pacific	*B*	EF025488	DQ900675
L. johnii (John's snapper)	Indo-West Pacific	*Y1*	DQ900720	DQ900682
		Y2	DQ900721	DQ900683
		Y3	DQ900722	EF376167
L. sebae (Emperor red snapper)	Indo-West Pacific	Q1	DQ900717	EF376168
		Q2	DQ900718	EF376169
		Q3	DQ900719	EF376170
L. erythropterus (Crimson snapper)	Indo-West Pacific	*H1*	EF025489	DQ900663
		H2	EF025490	DQ900664
		H3	DQ900707	DQ900665
L. malabarius (Malabar blood snapper)	Indo-West Pacific	*ML1*	EF025491	DQ900666
		ML2	DQ900708	DQ900667
		ML3	EF025492	EF025495
		ML4	DQ900704	EF376171
		ML5	DQ900705	EF376172
		ML6	DQ900706	EF376173
L. fulvus (Blacktail snapper)	Indo-Pacific	*JD1*	DQ900709	EF376174
		JD2	DQ900710	EF376175
		JD3	DQ900711	EF376176
L. fulviflamma (Dory snapper)	Indo-Pacific	*JY1*	DQ900712	DQ900680
		JY2	DQ900713	DQ900681
		JY3	EF376178	EF376177
L. vitta (Brownstripe red snapper)	Indo-West Pacific	*MH*	DQ900727	DQ900676
		MH1	DQ900730	EF376181
		MH2	EF025493	EF376182
L. ophuysenii (Spotstripe snapper)	Northwest Pacific	*MHE*	EF376183	DQ900677
		MHE1	DQ900725	EF376179
		MHE2	DQ900726	EF376180
Emmelichthys struhsakeri			AP004446	AP004446

chondrial genome special features in genome structure and evolution model (Gray 1989). Many complete mitochondrial genome sequences of marine species have been obtained, such as seabass (Lin et al. 2006), abalone (Maynard et al. 2005), eastern oyster (Milbury and Gaffney 2005), shrimp (Podsiadlowski and Bartolomaeus 2005), crustaceans (Miller et al. 2005), and so on. Mitochondrial genome sequences have been used extensively to investigate stock structure in a variety of fishes including chum salmon (Moriya et al. 2006), cod (Bakke and Johansen 2005), black bream (Burridge and Versace 2007), rockfish (Sotka et al. 2005), scallop (Nagashima et al. 2005), oysters (Klinbunga et al. 2005), and lobsters (Diniz et al. 2005). In this article, we compare complete DNA sequences of two mitochondrial genes, cytochrome *b* (*cyt b*) and cytochrome c oxidase subunit II (CO*II*), to examine phylogenetic relationships of 12 species of snappers occurring in SCS.

Materials and Methods

Specimens and Tissue Samples. A total of 38 individuals (Table 1) were used in the present analysis, including 37 snappers sampled near Zhanjiang from 2003 to 2005 (Figure 1) and one outgroup individual, *Emmelichthys struhsakeri* (Miya et al. 2003), also from the family Lutjanidae. All snapper individuals were classified according to morphological characteristics from FishBase, except that *L. ophuysenii* was classified according to Yukio et al. (1993). A few scales were taken from each individual with minimal invasion and stored at –85°C until DNA extraction. Type specimens of each species were preserved in 95% ethanol.

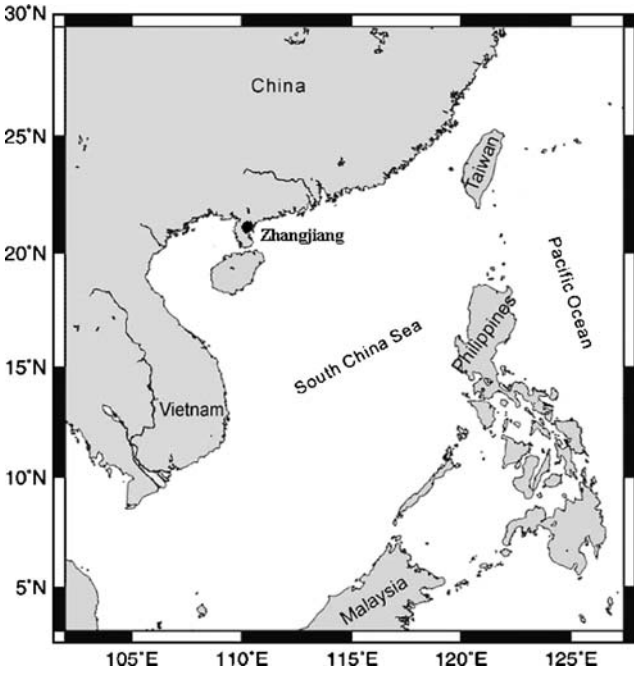

Figure 1. Sampling location of 37 snappers of genus *Lutjanus*. Black spot shows the Chinese city Zhanjiang near the sampling site, located in the north of South China Sea (SCS) in the western Pacific Ocean.

DNA Isolation, Amplification, Cloning, and Sequencing. DNA was isolated from approximately 0.005 to 0.010 g of scale (Sambrook et al. 1989). The following primer pairs were used to amplify mitochondrial genes: *cyt b*-forward 5′-GTG ACT TGA AAA ACC ACC GTT G-3′, *cyt b*-reverse 5′-CTC CAT CTC CGG TTT ACA AGA C-3′ (Song et al. 1998); CO*II*-forward 5′-CAA GCC AAC CAC ATA ACC-3′, CO*II*-reverse 5′-TCG GGA GTC ACC AGT CTT TA-3′ (designed from sequences of *Pterocaesio tile*, GenBank accession no. AP004447). Primers were synthesized by Sangon (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd.). Polymerase chain reaction (PCR) was performed in a final volume of 25 μl, including 5 to 10 ng of each template DNA, 2.5 μl of 10× PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 2.0 μl of 1.5 mM $MgCl_2$, 2.5 μl of 2.5 mM dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP), 50 pmol of each primer, 0.5 μl of *Taq* DNA polymerase (2.5 U/μl). All the above reagents were commercially available from Sangon. PCR fragments were generated with the following cycle parameters: initial denaturation (94°C for 80 s); 30 cycles of reactions [denaturation at 94°C for 42 s, annealing at 47°C (*cyt b*) or 50°C (CO*II*) for 45 s; extension at 72°C for 60 s]; final extension (72°C for 300 s); icebox (4°C) indefinitely and PCR fragments cloned into pUCm-T vector (Bio Basic Inc.), ligated vectors were transformed and cloned into DH5α competent cells, and the cells cultured on Luria-Bertani (LB)/ampicillin plates with IPTG and X-gal (Promega) overnight. Positive colonies were sequenced with ABI 377 automated DNA sequencer (Applied Biosystems) using the Big Dye Terminator Cycle Sequencing Reaction Kit and M13 primers.

Data Analysis. Nucleic acid sequences were aligned by the CLUSTAL W feature with Alignment Explorer of MEGA version 3.1(Kumar et al. 2004) using *E. struhsakeri* (GenBank AP004446) as a reference sequence, and were translated into amino acid sequences to test for presence of pseudogenes. To test the potential from intraspecific variation, three individuals from each species were sequenced with the exception of two-spot red snapper and Malabar blood snapper (one and six individuals, respectively). Various parameters [e.g., α parameters in a γ distribution (α), pattern of nucleotide substitution (s/v) and

saturation (Iss and Iss.c)] were estimated via DAMBE (Xia 2000; Xia and Xie 2001) for use in further analyses. The homogeneity of the base composition was tested via the ID-Test with the MEGA program. For phylogenetic analysis, sequences of both genes were combined (CO*II* and *cyt b*, 1831 bp), and neighbor-joining (NJ) tree and maximum parsimony tree recovered with the MEGA, using *E. struhsakeri* as the outgroup. Bootstrap values for NJ and MP tree were estimated with searches using 1000 replicates.

Results

Sequence Analysis. The complete *cyt b* and CO*II* genes were 1141 bp and 690 bp long, respectively. Most of the parsimony informative sites (*cyt b*: 257, CO*II*: 123) were found at the third codon position (*cyt b*: 229, CO*II*: 113). The mean base contents of *cyt b* and CO*II* were 0.25 A, 0.33 C, 0.15 G, 0.27 T and 0.29 A, 0.29 C, 0.16 G, 0.26 T, respectively. Anti-G biases were detected at the second and third positions in both genes, typical of mitochondrial genes encoded on the H strand (Johns and Avise 1998). Nucleotide substitution was biased in favor of transitions (*cyt b*: 620, CO*II*: 343) over transversions (*cyt b*: 330, CO*II*: 87), resulting in s/v ratios of 1.9 and 3.9.

Phylogenetic Analyses Estimates of interspecific sequence divergence ranged from 0.05% (H1 of *L. erythropterus* and Z3 of *L. argentimaculatus*) to 15.25% (*L. sebae* and *L. fulviflamma*). Intraspecific divergence varied from 0.00% to 4.92%, with the largest value within *L. erythropterus* greater than many interspecific distances. The topology generated by NJ analysis (Figure 2) was similar to the topology inferred using parsimony. Monophyly of seven species (*L. russellii, L. stellatus, L. bohar, L. johnii, L. sebae, L. fulvus*, and *L. fulviflamma*) was strongly supported by bootstrap analysis; however, unexpectedly high similarities were found between *L. vitta* and *L. ophuysenii* (clade 1), and between H1 of *erythropterus* and *L. argentimaculatus* (clade 2).

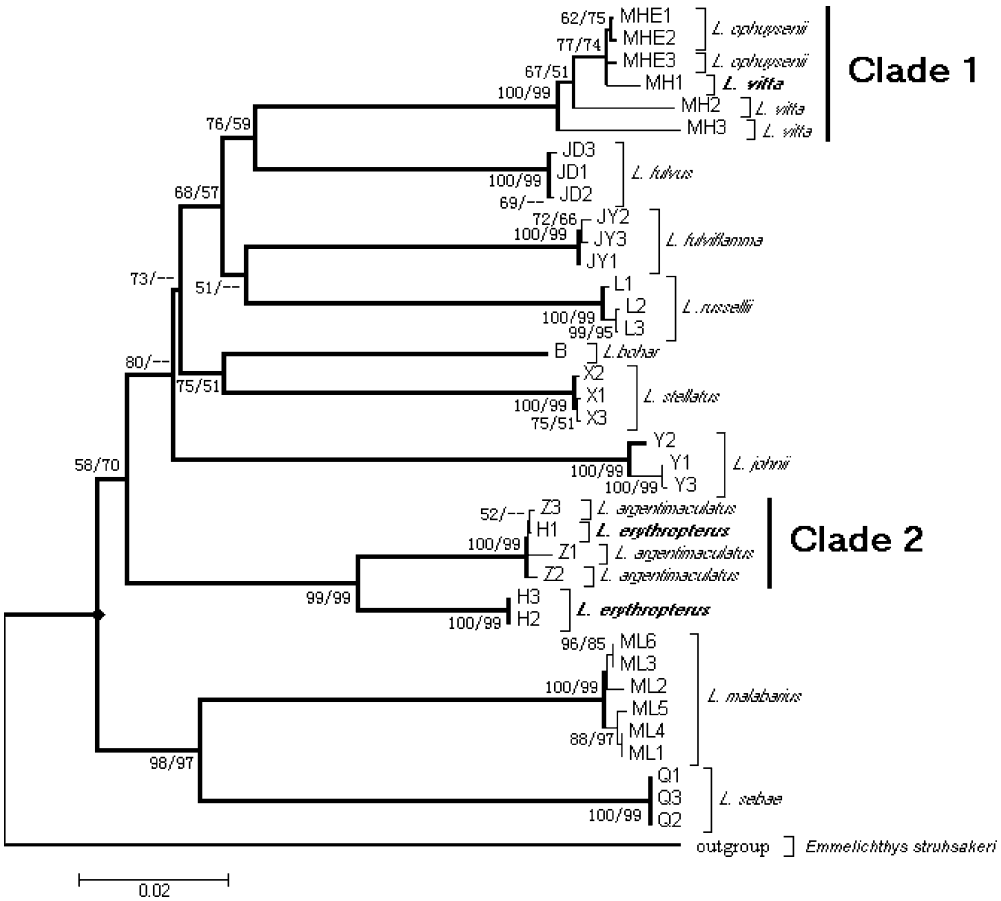

Figure 2. Neighbor-joining (NJ) consensus tree of snapper species present in South China Sea rooted by *Emmelichthys struhsakeri*. Neighbor-joining and maximum parsimony bootstrap values higher than 50% are indicated at nodes (NJ/MP).

Discussion

It is very useful and convenient to test and revise the morphology-based system with the mtDNA data (Thomas et al. 2002; Utter and Allendorf 1994). It has been shown that longer sequences are preferable, especially if we consider the restrictions in mutation imposed on protein-coding genes (Martin et al. 1990). In the present study, analyses were performed using a combined data set of *cyt b* and CO*II* mitochondrial genes. The strong correlation in topology of trees inferred by MP and NJ methods demonstrates the high level of accuracy of the phylogenetic reconstruction carried out in this survey. For practical purposes, we sampled all individuals at the same site near Zhanjiang, located in northwestern SCS. We acknowledge the possible existence of interlocal variation but it is likely not important. These coral reef species with pelagic larvae are genetically similar over broad regions like the Gulf of Mexico and SCS, and the high levels of intraspecific homogeneity of *Lutjanus* are well supported by several population structure studies (Garber et al. 2004; Zhang et al. 2006).

This phylogenetic analysis was based on sequences of *cyt b* and CO*II* mitochondrial genes, with more individuals from each species than in previous studies (Sarver, 1996; Zhu et al. 2006). The analysis with more individuals not only minimizes the potential of intraspecific variation, but also reveals interactions between close species. For example, it indicates that *L. vitta* is paraphyletic (Figure 2), providing direct evidence of a close relationship with *L. ophuysenii*. Given the overlapping habitat in SCS, we infer that *L. vitta* and *L. ophuysenii* are still in the statues of species flock in SCS with the ability of nature hybridization like some coral reef fish (Yaakub et al. 2006).

L. argentimaculatus and *L. erythropterus* are important species in aquaculture (Russell 2003), which can be differentiated by the vertical band (Table 2). The individuals of *L. argentimaculatus* and *L. erythropterus* cluster in corresponding lineages with high bootstrap value except for H1 of *L. erythropterus*. Laboratory hybridization experiments for the genus *Lutjanus* indicate that several species may interbreed (Domeier and Clarke 1992; Loftus 1992,Chen et al. 2006); therefore, observed patterns of variation may reflect introgressive hybridization between *L. argentimaculatus* and *L. erythropterus*.

In this study, the phylogenetic relationships from mtDNA data are consistent with Allen's morphology-based system (Table 2). Body depth and band pattern show stronge correlation with topologies, consistent with other coral reef fish (Yaakub et al. 2006), and also correspond well to the grouping of species in the Allen's morphology-based system rather than that of Cheng. For instance, *L. argentimaculatus*, *L. erythropterus, L. malabarius*, and *L. sebae* have close affinities (Figure 2 and Table 2). Morphologically, the body colors of these four species adults are red and their bodies are relatively deep; in addition, the body sizes of adults are much larger among the genus *Lutjanus*. This issue is congruent with that obtained using the molecular data from AFLP (Zhang et al. 2006).

The present analysis (Figure 2 and Table 2), analysis of mtDNA-RFLP (Wang et al. 2005), and microsatellite DNA markers (Liu and Liu 2006) support that *L. vitta* and *L. ophuysenii* are the closest relatives. In contrast, allozyme data indicated *L. vitta* is well separated from *L. ophuysenii* and *L. russellii* (Lee and Cheng 1996). Lee and Cheng (1996) emphasized that morphologic characters showing differences included the dorsal fin ray formulae [X, 12–13 (mostly 13) in *L. ophuysenii*

Table 2. Relationship between phylogenetic tree and appearance characteristics

Nj tree	Science name	English name	Appearance pattern					
			D.B.	H.B.	V.B.	W.S.	B.S.	R.C.
100	*Lutjanus vitta*	Brownstripe red snapper	No	Yes	No	No	No	Yes
76	*L. ophuysenii*	Spotstripe snapper	No	Yes	No	No	Yes	Yes
68	*L. fulvus*	Blacktail snapper	No	Yes	No	No	No	No
51	*L. fulviflamma*	Dory snapper	No	Yes	No	No	Yes	No
73	*L .russellii*	Russell's snapper	No	Yes	No	No	Yes	No
80	*L. stellatus*	Star snapper	Yes	No	No	Yes	No	No
75	*L. bohar*	Two-spot red snapper	Yes	No	No	Yes	No	Yes
58	*L. johnii*	John's snapper	*No*	*No*	*No*	*No*	*Yes*	*No*
99	*L. argentimaculatus*	Mangrove red snapper	Yes	No	No	No	No	Yes
	L. erythropterus	Crimson snapper	Yes	No	*Yes*	*No*	*No*	*Yes*
98	*L. malabarius*	Malabar blood snapper	Yes	No	*Yes*	*No*	*No*	*Yes*
	L. sebae	Emperor red snapper	Yes	No	*Yes*	*No*	*No*	*Yes*
	Emmelichthys struhsakeri		No	No	No	No	No	Yes

D.B. = Deep body; H.B. = horizontal band; V.B. = vertical band; W.S. = white side spot; B.S. = black side spot; R.C. = red color. *L. sebae* have three dark red vertical bands. *L. russellii, L. fulvus*, and *L. fulviflamma* have several horizontal bands, but the bands of *L. russellii*

and *L. vitta* and X, 14–15 (mostly 14) in *L. russellii*]; minute scales on the lower preoperlular flange were present only in *L. vitta*; the preopercular notch was shallow in both *L. ophuysenii* and *L. russellii*, but deeper in *L. vitta*; an ovoid black spot on the body side was present in both *L. ophuysenii* and *L. russellii*, but absent in *L. vitta*. Given that the interspecific relationships are consistent with Allen's morphology-based identifications, we think that compared with *L. russellii*, the wider and longer pattern of stripes and red body color of *L. ophuysenii* (Spotstripe snapper) and *L. vitta* (brownstripe red snapper) warrant recognition.

According to the characteristics described by Allen (1985), we sampled individuals of *L. malabaricus* in Zhanjiang, and all individuals were identified from *L. erythropterus* easily by the opposite characteristics that "a prominent black band runs across the caudal peduncle with a pearly-white border" in *L. malabarius*. NJ tree by mtDNA data (Figure 2) show that individuals from *L. malabaricus* and *L. erythropterus* belong to two different lineages in SCS: All six individuals of *L. malabaricus* clustering together closely are sister to the *L. sebae* clade; however, three individuals of *L. erythropterus* are very close to the individuals of *L. argentimaculatus*. Thus, both morphologic and molecular data are consistent with the conclusion that *L. malabaricus* is a valid species of genus *Lutjanus* in SCS.

In conclusion, the present study could serve as a reference in attempts to resolve relationships among common species of genus *Lutjanus* in SCS and justify the taxonomic status of *L. malabaricus* in mainland of China. However, it is necessary to acquire more evidence for verification of the phylogenetic relationships and putative hybrid status.

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