

Phylogenetic Relationships of Emperors (Lethrinidae) and Snappers (Lutjanidae) in Vietnam based on Mitochondrial DNA Sequences

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Abstract— Fishes of the families Lethrinidae (emperors) and Lutjanidae (snappers) are commercially important in South China Sea. However, in terms of morphology, they can be confused because of a basic “fish-like” shape with large mouths and notched tails. In Vietnam, however, classification system of fish species based primarily on morphological characteristics. Therefore, the number and distribution of many species is still controversial. The purpose of this study was to clarify the phylogeny of commercial fish species of the genus *Lethrinus* and *Lutjanus* distributed in Vietnam. Currently 4 lethrinid and 4 lutjanid species are described based on morphologic characters. Combine with Genbank sequences, a phylogenetic tree was constructed based on 16S gene of mitochondrial DNA using Maximum parsimony, maximum likelihood and Bayesian inference approaches. The phylogram showed that two families exhibit monophyly with differences of species range sequence from 1,9% to 3,9% in *Lethrinus* and from 1,9% to 4,4% in *Lutjanus*. Additionally, 16S sequences of current species were identical to sequences of the same species reported in Genbank. Continue sampling of commercial fish species and classification based on morphology and genetics to build DNA barcoding of fish species in Vietnam is really necessary.

Keywords— Lethrinidae, Lutjanidae, 16S rRNA, morphology, phylogeny

I. INTRODUCTION

FISHES of the families Lethrinidae (emperors) and Lutjanidae (snappers) are commercially important in Vietnam Eastern Sea. Most lethrinids and lutjanids are carnivorous and occur in reef or other structure associate habitats where they feed primarily on fishes, crustaceans, molluscs and pelagic urochordates [1]. In addition, they are long-lived, slow-growing, and important to fisheries, providing a significant food resources for developing countries [2].

The family Lutjanidae currently include 123 species in 21 genera and 6 subfamilies [3], genus *Lutjanus* possess the highest species diversity with 69 species. The Lethrinidae include 39 species in 5 genera, with 29 species in the most common genus *Lethrinus* [4]. In terms morphological, Allen

(1985) used body shape, body color, stripes, spines and the rays of the dorsal fin as primary distinguishing characteristics [5]. Cheng (1987) used the scales around the lateral line and the opercula bones as specific taxonomic characteristics [6]. However, accurate phylogenetic relationships remain unclear. Due to their morphologic confusing, the phylogeny of some species of lutjanids and lethrinids have recently been examined using molecular data. Monophyly of 27 species of common Indo-Pacific snappers was supported using the 16S ribosomal RNA (rRNA) and cytochrome b (cyt b) genes [7]. Zang and Liu (2006) studied phylogeny of Lutjanids using Amplified Fragment Length Polymorphism (AFLP) analysis of genome DNA and mitochondrial 12S rRNA gene. Result indicated that the genus *Lutjanus* is a monophyletic group, whereas the phylogenetic relationships of *Pristipomoides*, *Caesio* and *Pinjalo* still remains uncertain [8]. Alicia et al. (2002) studied the evolution of the primary feeding types in 20 species of *Lethrinus* using complete cyt b gene sequences. Result showed *Lethrinus* species divided into two clades. One of these clades exclusively contains low-bodied species with conical teeth while the other clade only comprise the high-bodied species with molariform teeth [9].

Nguyen Nhat Thi and Nguyen Van Quan (2004) reported 20 species, 6 genera (*Aphareus*, *Lutjanus*, *Macolor*, *Pristipomoides*, *Symphorus* and *Symphorichthys*) in Lutjanidae, and 15 species, 4 genera (*Gnathodentex*, *Gymnocranius*, *Lethrinus* and *Monotaxis*) in Lethrinidae of Spratly Archipelago [10]. Nguyen Van Quan (2013) reported 4 species, 2 genera (*Caesio* and *Lutjanus*) belong to Lethrinidae in total 58 species of reef fishes from marine water of Bach Long Vi islands, Vietnam [11].

Above information is relatively fragmented and not entirely reflect the classification system of fish species in Vietnam. Moreover, these survey data are based primarily on morphological characteristics, which can lead to confusion.

In the present study, species identification was confirmed by morphological and genetic characters; phylogeny of the genus *Lethrinus* and *Lutjanus* was clarify based on 16S rDNA sequences. These data contribute to the DNA barcoding of marine fish species in Vietnam, as the basis for the management and conservation of natural resources.

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II. MATERIALS AND METHODS

A. Sampling and identification

Emperor and snapper fish were collected along the coast of Viet Nam from the South (Phu Quoc, Bac Lieu and Vung Tau) and Central (Khanh Hoa and Da Nang). The samples were transferred to the laboratory keeping in alcohol or on ice and then store at -40°C before analysis.

For morphological analysis, all specimens were identified based on taxonomic characters such as body and fins colour, the presence or absence of scales on the cheek, number of spine and soft rays belong to dorsal, anal, ventral, pectoral and caudal fin (Fig. 1). Species was identified following Allen, (1985); Carpenter and Allen, (1989) [5] [12].

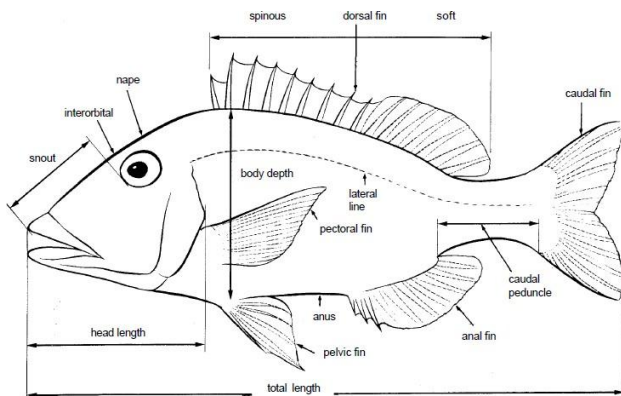


Fig. 1 External morphological characters [12]

B. DNA extraction, PCR amplification and sequencing

Total DNA was extracted from the muscle of each individual using GeneJET Genomic DNA Purification Kit (Thermo scientific) following to the manufacturer's instructions. 5μ of the lysate was used as template in PCR reactions to amplify the 16S gene of mitochondrial DNA (16S mtDNA), using the primers 16Sar $5'$ -CGCTGTTTATCAAAAACAT- $3'$ and 16Sbr $5'$ -CCGGTCTGAACCTCAGATCACGT- $3'$ [13].

PCR reactions were performed using a total volume of $50\mu\text{l}$ including 20ng DNA template, $5\mu\text{l}$ 10X Dream Taq buffer (Fermentat), 0.25 nM each dNTP, 0.2 pM each primer, 1 unit of Taq polymerase ($5\text{U}/1\mu\text{l}$) and distilled water to the final volume. Biorad thermocyclers (Icycler) were used under the following temperature program: initial denaturation 94°C for 3 min, followed by 35 cycles of denaturation 94°C for 30s, annealing 48°C for 30s and final extension at 72°C for 5 minutes. PCR products were electrophoresed on 1.5% agarose gel stained with ethidium bromide, and bands were visualized under a UV transilluminator.

The PCR products were purified using a PCR clean up system kit (Promega), and pre-sequenced using dye – labels dideoxy terminator (Big Dye Terminator v. 3.1, Applied Biosystems) with the same primer as the PCR reaction at the following temperatures: 96°C for 30s, 50°C for 30s and 60°C for 4 min. Products were sequenced in ABI Prism 3.700 DNA Analyser (Applied Biosystems).

Sequence contigs were assembled using Geneious v. 7 (<http://www.geneious.com/>). The resulting sequences were confirmed by the Basic Logical Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/>). Sequences were

initially aligned by eye using the sequence editor BioEdit 7.0 [14].

C. Phylogenetic analysis

Sequences in this study together with sequences of other lethrinid and lutjanid species available from Genbank were used in the phylogenetic analysis (Table 1).

Data were analysed using 3 approaches, i.e., Neighbour joining (NJ), maximum parsimony (MP) and Bayesian inference (BI). NJ analyses were conducted from MEGA 6 under 1000 replicate. MP analysis were conducted using PAUP* 4.0 [15]. Bootstrap support values of the MP analysis were used to assess the robustness of the findings. Bootstrap support values were computed from 1,000 replicates randomized 10 times with tree-bisection-reconnection (TBR) addition sequence.

Prior to BI analyses, best-fit models of nucleotide substitution were selected by the Akaike Information Criterion as implemented by MrModeltest 2.2 [16]. Bayesian analyses were conducted in MrBayes 3.1.2 under the selected best-fit models and parameters. Numbers at the interior branches of the majority-rule consensus tree present posterior probability (PP). Tree display and editing were performed in TreeView 1.6.6 [17].

TABLE 1
GENBANK ACCESSION NUMBERS FOR 16S MTDNA GENES

Family	Species	Accession No.	
Lethrinidae	<i>Lethrinus lentjan</i>	Present study	
	<i>L. ornatus</i>	Present study	
	<i>L. atkinsoni</i>	Present study	
	<i>L. rubrioperculatus</i>	Present study	
	<i>L. haematopterus</i> GB	JN688793.1	
	<i>L. obsoletus</i> GB	AF247446.1	
	<i>L. harak</i> GB	JQ938963.1	
	<i>L. nebulosus</i> GB	AB793300.1	
	<i>L. ornatus</i> GB	AF247446.1	
	<i>L. rubrioperculatus</i> GB	AF247447.1	
	<i>L. lentjan</i> GB	DQ532896.1	
	Lutjanidae	<i>Lutjanus russelli</i>	Present study
		<i>L. argentimaculatus</i>	Present study
		<i>L. monostigma</i>	Present study
		<i>L. gibbus</i>	Present study
		<i>L. sebae</i> GB	DQ444485.1
		<i>L. stellatus</i> GB	DQ444483.1
<i>L. fulviflamma</i> GB		DQ444479.1	
<i>L. erythropterus</i> GB		DQ444480.1	
<i>L. lunulatus</i> GB		JX192596.1	
<i>L. fulvus</i> GB		DQ444486.1	
<i>L. quinquelineatus</i> GB		DQ784736.1	
<i>L. kasmira</i> GB		DQ784734.1	
<i>L. carponotatus</i> GB		DQ784730.1	
<i>L. bohar</i> GB		DQ784729.1	
<i>L. johmii</i> GB		DQ444484.1	
<i>L. rivulatus</i> GB	AP006000.1		
<i>L. malabaricus</i> GB	NC_012736.1		
<i>L. russelli</i> GB	DQ444478.1		
<i>L. argentimaculatus</i> GB	DQ444481.1		
<i>L. monostigma</i> GB	DQ784735.1		
<i>L. gibbus</i> GB	DQ784733.1		

III. RESULTS AND DISCUSSION

A. Species identification

❖ Genus *Lethrinus*:

Genus *Lethrinus* (Lethrinidae) has body oblong, covered with small ctenoid scales. Mouth size moderate. Cheek without scales. Dorsal fin continuous with X spines and 9 soft rays; anal fin with III spines and 8 soft rays; pelvic fins with I spines and 5 soft rays; pectoral fin with 13 soft rays; caudal fin moderately forked. Lateral line complete.

- *Lethrinus lentjan* Lacepède, 1802 – Redspot emperor

Body greenish, becoming paler below. Pectorals fins are yellow, the pelvic and anal fins are orange with a reddish margin. **The posterior margin of the opercle and near base of the pectoral fin are red** (Fig. 2A).

- *Lethrinus ornatus* Valenciennes, 1830 - Ornate Emperor

Body color is dusky whitish, becoming lighter below, with **four orange stripes**. **The posterior edges of the operculum and preoperculum are bright red**. The head is brown, a red spot on the lower front edge of the eye. The pectoral fin is orangish, the pelvic, anal and most of the dorsal fin is whitish. The edge of the dorsal and caudal fin is reddish (Fig. 2B).

- *Lethrinus rubrioperculatus* Sato, 1978 - Spotcheek emperor

Body color is brown, with scattered irregular small black blotches. **The upper edge of the operculum usually has a red spot**. **Lips are usually red**. Fins are pinkish (Fig. 2C).

- *Lethrinus atkinsoni* Seale, 1910 - Pacific yellowtail emperor

Color is yellowish on the upper sides and white ventrally. The head is brown, lips reddish, caudal peduncle yellowish. **The base of pectoral fins, the basal membranes of the anal fins and the edges of the pelvic, dorsal, anal and caudal fins are often reddish** (Fig. 2D).

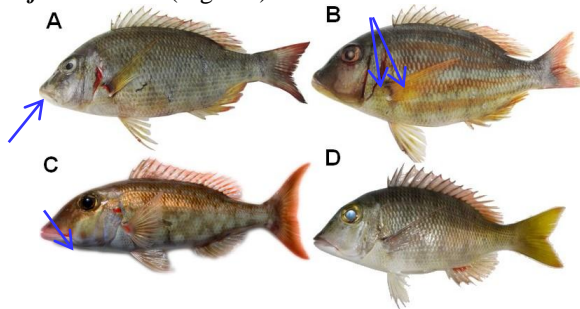


Fig. 2 Emperor of the family Lethrinidae from Viet Nam: A. *Lethrinus lentjan*; B. *Lethrinus ornatus*; C. *Lethrinus rubrioperculatus*; D. *Lethrinus atkinsoni*. Blue arrows show the specific characters.

❖ Genus *Lutjanus*:

Genus *Lutjanus* has similar characters with *Lethrinus*, but they have some special characters such as dorsal fin continuous with a slight notch between the spinous and soft portions, with soft rays in dorsal fin 11 – 16. Pectoral fins have 15 to 18 rays. Most species have scales on cheek.

- *Lutjanus russellii* Bleeker, 1849 - Russell's Snapper

Body color is grey. **There is a black spot above the lateral line below the soft part of the dorsal fin. Pectoral and anal fins are yellow** (Fig. 3A).

- *Lutjanus gibbus* - Humpblack red snapper

Humpblack red snapper has a pinkish-grey to red body. The fins are red. **The soft dorsal, caudal and anal fins have narrow white margins** (Fig. 3B).

- *Lutjanus monostigma* Cuvier, 1828 – One-spot snapper

It has a grey body on the upper sides and yellow ventrally. **All fins are yellow**. It has **a black spot below the middle of the dorsal fin that is intersected by the lateral line** (Fig. 3C).

- *Lutjanus argentimaculatus* Forsskal, 1775 - Mangrove red snapper

Coloration of the mangrove red snapper is reddish. Juveniles with a series of about eight whitish bars crossing sides and 1 to 2 blue lines across cheek (Fig. 3D).

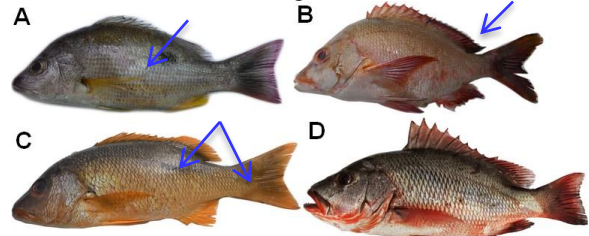


Fig. 3 Snapper of the family Lutjanidae from Viet Nam: A: *Lutjanus russellii*; B: *Lutjanus gibbus*; C: *Lutjanus monostigma*; D: *Lutjanus argentimaculatus*. Blue arrows show the specific characters.

B. Phylogenetic analysis

The 16S dataset consists of more than 600 bp, of which 546 bp were unambiguously aligned. Tree topology from the MP method (Fig.4) was similar to that of the best NJ tree and showed only minor differences from the BI tree.

The phylogenetic tree included two main clades with high BT support, correspond to the genus *Lutjanus* and *Lethrinus*, respectively. In clade 1, there were five strongly supported sub-group. **Group I** include *Lutjanus russellii*, *L. carponotatus*, *L. fulviflamma*, *L. fulvus* and *L. monostigma*. Generally, morphology characters such as red-grey coloration of these five species suggest a close relationship, however *L. carponotatus* and *L. fulvus* lack the distinct black spot near the lateral line below the soft dorsal fin seen in the latter three species. **Group II** include *Lutjanus bohar* and *L. gibbus*. According to Terrence and Thomas (2006) [7], this relationship is not strongly supported as *Lutjanus bohar* and *L. gibbus* vary in morphologically diagnostic characters: dorsal rays X, 13 and 14; anal rays III, 8; pectoral rays 16 and 17; lateral line scales 48 – 51 and 47 – 51, respectively. **Group III** include 5 species, among them, *L. quinquelineatus* and *L. kasmira* having several blue lines on the body, while these lines lacking on later species *L. stellatus*, *L. rivulatus*, *L. lunulatus*, **Group IV** include *L. argentimaculatus*. Among *Lutjanus* species, only juveniles and young adult of *L. argentimaculatus* enters freshwater environment, this may be a reason why *L. argentimaculatus* clustered in one separate sub-group. **Group V** include *L. johnii*, *L. sebae* and *L. erythropterus*. With these species, body turn on to red color in the adult stage, as well as their bodies relatively deep [18]. Miller and Cribb (2007) reported the similar phylogenetic relationships, and mentioned *L. johnii* of having the black spot similar to species of group I should be stayed in the same clade [7].

Clade 2 was subdivided into two groups with high node support (MP 100%, PP 100%, MJ 100%). One of these groups

(Group I) consists of *Lethrinus rubrioperculatus* and *L. haematopterus*. These species are low-bodied forms with conical teeth. The second group (Group II) includes *L. ornatus*, *L. lentjan*, *L. harak*, *L. atkinsonii*, *L. nebulosus* and *L. obsoletus*. These species are characterised by high-bodied forms with molariform or submolariform teeth [9], [19].

Current research provides key identification for common emperors and snappers in Vietnam. Formal description, voucher preservation and DNA barcoding could help update species checklist in Vietnam. Furthermore, molecular phylogeny of these species performed morphophyly of two genera *Lethrinus* and *Lutjanus* with diagnostic taxonomic characters, which in concordant to previous studies [7].

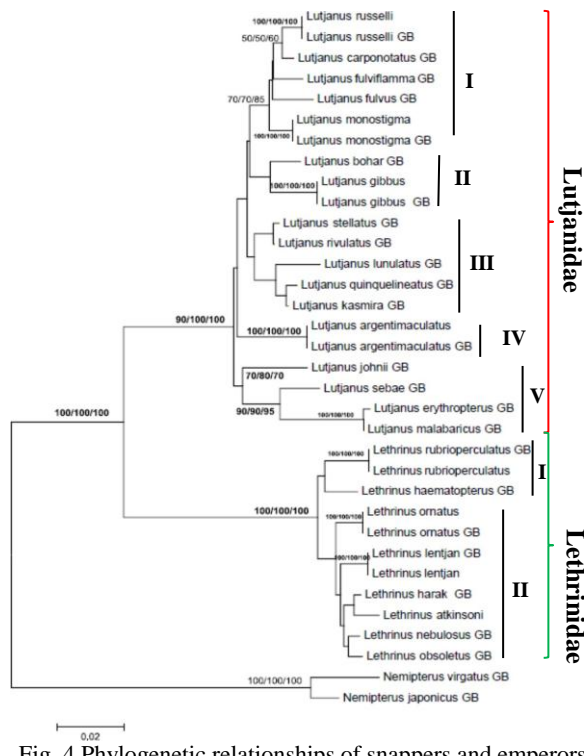


Fig. 4 Phylogenetic relationships of snappers and emperors based on the 16S rDNA. MP bootstrap value, posterior probability (BI analysis) and bootstrap value from NJ analysis along the branch. *Nemipterus virgatus* and *N. japonicus* were used as group.

IV. CONCLUSION

4 lethrinid and 4 lutjanid species in Viet Nam were morphological identified: *Lethrinus lentjan*, *Lethrinus ornatus*, *Lethrinus rubrioperculatus*, *Lethrinus atkinsoni*, *Lutjanus russellii*, *Lutjanus gibbus*, *Lutjanus monostigma*, *Lutjanus argentimaculatus*. Phylogram constructed based on 16S mtDNA sequences indicates the monophyly of genus *Lethrinus* and *Lutjanus*, strongly support specific taxonomic subgroups.

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