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Research Article

Evolutionary Lineages in Genus *Lethrinus* (Family: Lethrinidae) and the Corresponding Trophic Evolution Based on DNA Barcoding

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Abstract

Background: The present study aimed at studying the phylogenetic relationships of 18 *Lethrinus* species and their associated trophic evolution on the basis of their filtered and validated DNA barcoding (cytochrome oxidase subunite I (COI)-sequences) released by the BOLD system database. **Materials and Methods:** About 98 COI sequences of 18 *Lethrinus* species were retrieved from BOLD system released nucleotide database and analyzed using different phylogenetic softwares. **Results:** Intra and inter-specific variations in COI-sequences were recorded with emphasis on geographic variations. Some COI-sequences were postulated to be of misidentified specimens and others were questionable leading to paraphyla. The COI-based phylogenetics using different statistical methods established two lineages in genus *Lethrinus* with *L. haematopterus* as ancestor group. These two lineages were corresponding with two obvious trophic types: Low-bodied species with conical lateral teeth and high bodied species with molariform teeth with the sister group *L. haematopterus* of high-bodied species with conical teeth. **Conclusion:** These findings emphasizes on the higher validity of DNA barcoding in discovering phylogenetics of genus *Lethrinus* in comparison to those based on cytochrome b.

Key words: DNA barcode, *Lethrinus*, trophic evolution, phylogenetics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lethrinids are taxonomically considered one of the most problematic group of tropical marine fish families. Their identification-related problems are primarily due to their relatively constant and conservative morphological and anatomical characteristics especially on the specific level[1,2]. The Lethrinidae has been classified with three other families into the superfamily Sparoidea in the phyletic sequence Nemipteridae, Lethrinidae and Sparidae plus Centracanthidae[3,4]. Different studies are in concern of the evolutionary relationships between these families[3,5,6]. Carpenter and Johnson[3] placed on morphological bases, Nemipteridae as the sister group of Lethrinidae and Sparidae (including centracanthids).

According to Carpenter and Allen[1], the Lethrinids are classified into two subfamilies: Lethrininae and Monotaxinae based on head scalation patterns and dorsal and anal-fin ray counts. Lo Galbo *et al.*[2] reported that the Lethrininae includes *Lethrinus* with 29 species and the other four genera, *Gnathodentex*, *Gymnocranius*, *Monotaxis* and *Wattsia* comprise the Monotaxinae; all of monotaxine genera are monotypic except *Gymnocranius*. These researchers stated that for *Lethrinus* with very conservative morphological characters in particular, an independent data set is necessary to construct a phylogeny; cytochrome b gene was considered. Other researchers tried to assess some interspecific relationships in *Lethrinus* species based on scale morphology, morphometris, biology, isoenzymes and osteology in Egypt and Saudi Arabia[7-10].

Piscivores, benthic invertebrate carnivores, zooplanktivores and herbivores are among the wide range of trophic types of sparoid fishes which are common components of hard bottom demersal fish communities with exception of *Nemipterus*[3]. The studies of evolution of the trophic types especially in the closely related fishes are rare[2,11] and the reliable morphological characters in this concern are dentition and body shape, which are correlated with feeding type[2,3,12]. Carpenter[12] studied the relationship of dentition, body shape and the associated specific feeding mode of species of *Lethrinus* with emphasize on their clues to the evolution of these feeding types. The *Lethrinus* species are demersal feeders with three distinct modes, low-bodied species with conical lateral teeth (LC), high bodied species with molariform (HM) teeth and high-bodied species with conical teeth (HC)[2]. According to researchers, the *Lethrinus* species with first two modes are mesocarnivore stalker and specialists, respectively and those with the latter mode are mesocarnivore generalists. Hanel and Sturmbauer[11] hypothesized that the same trophic types evolved multiple time within the family. Lo Galbo *et al.*[2] and Orrell *et al.*[13] generated comprehensive phylogeny of sparidae genera and *Lethrinus* based on cytochrome b sequences and in concern with trophic types. Use of a more conservative gene may be necessary to adequate phylogeny of these genera.

Variable DNA sequence analyses have been reviewed[14,15] and used for three decades to assist species identifications for different taxonomic groups in different laboratories[16-23]. This DNA-based approaches including DNA Barcoding (mitochondrial cytochrome c oxidase subunit I, COI) are used to identify fishes and resolve taxonomic problems including the discovery of new/cryptic species[24-27]. The DNA barcoding has been utilized to evaluate the incidence of fish species substitutions in North America[28], Europe[29] and Italy[30,31]. Due to its lower effective population size with rapid rate of evolution, various genes of mtDNA genome are applied to investigate different issues[22,32-36]. Meyer[37] stated that different parts of the mitochondrial gene are known to evolve at different rates. The DNA barcoding methodology requires intraspecific DNA barcode variation substantially lesser than the interspecific one for accurate identification of individuals. One of the criticisms reported by many researchers including Lipscomb *et al.*[38], Moritz and Cicero[39] and Dasmahapatra and Mallet[40] was that samples generally taken from a narrow geographic region may markedly lead to underestimated intraspecific level of variability.

Hebert *et al.*[24] proposed the use of the mitochondrial DNA gene COI as a global bio-identification system for animals with empirical support extended from studies of invertebrates to birds[19,41]. Due to criticisms on such approach, Collins and Cruickshank[42] referred to 7 deadly sins which are represent serious limitation to the utility of DNA barcoding especially in creating reference libraries. Becker[43] also identified some of the human errors as the primary source of error in FISH-BOL barcode data. Spouge and Marino-Ramirez[44] described a workflow for measuring the efficacy of DNA barcode in identifying species including the probability of correct identification.

According to the aforementioned findings, the present work aimed at determination of the evolutionary lineages in genus *Lethrinus* (Family: Lethrinidae) using their well-identified DNA barcodes (Cytochrome c oxidase subunit I, COI) recorded and released in the BOLD-system database and to examine the evolution of the three primary feeding types in *Lethrinus* species in comparison with the study of Lo Galbo *et al.*[2] based on cytochrome b gene sequences. The 18 *Lethrinus* species used include *L. lentjan*, *L. obsoletus*, *L. ornatus*, *L. crocineus*, *L. nebulosus*, *L. harak*, *L. mahsena*,

L. atkinsoni, *L. laticaudis*, *L. semicinctus*, *L. ravus*, *L. conchyliatus*, *L. rubrioperculatus*, *L. microdon*, *L. xanthochilus*, L. sp., *L. olivaceus*, *L. sminiatus* and *L. haematopterus* . Moreover, the origin of these species was also considered in their range of distribution.

MATERIALS AND METHODS

Data source: About 98 COI sequences of 18 *Lethrinus* species (Table 1) were retrieved from BOLD system released nucleotide database[45]. These *Lethrinus* species include *L. lentjan*, *L. obsoletus*, *L. ornatus*, *L. crocineus*, *L. nebulosus*, *L. harak*, *L. mahsena*, *L. atkinsoni*, *L. laticaudis*, *L. semicinctus*, *L. ravus*, *L. conchyliatus*, *L. rubrioperculatus*, *L. microdon*, *L. xanthochilus*, L. sp., *L. olivaceus*, *L. miniatus* and *L. haematopterus*. The L. sp., as referred in the database did not have a scientific name and is considered as it is. These species represent different geographical regions, India, Japan, Australia, China, Taiwan, Iran, South Africa, Madagascar and Mozambique. Moreover, different released sequences of different species of *Gymnocranius* (Lethrinidae), *Acanthopagrus* (Spariidae), *Acanthurus* (Acanthuridae) and *Lophius* (Lophiidae) from the same database were retrieved to work as outgroups in current analysis (Table 2). The specimen used in COI-sequence preparations were collected in different times and their sequences are published (BOLD system database).

Sequence and haplotype analyses: The COI-sequences of *Lethrinus* species in concern are analyzed using different softwares including MEGA 6[46] with Clustal W, Arlequin 3.5[47], DnaSP 5.10[48], BioEdit 7.1.9[49] and SplitTree4[50]. The outputs and parameters estimated by these softwares include aligned nucleotide sequences, Kimura 2-parameter (K2P) distances (overall mean, between mean and within mean species distances), phylogenetic trees and split tree, nucleotide composition, G+C content, numbers of polymorphic sites, parsimony informative sites, mutation and haplotypes,

Table 1: BOLD-system ID, GenBank_Accession ID collection locality and trophic catogeries for released DNA barcodes COI-sequences of different *Lethrinus* species used in the present study

BOLD-system ID	Species	Trophic status**	Country	GenBank accession
DSFSE392-08	*L. rubrioperculatus*	CL	South Africa	JF493757
TZMSA139-04	*L. rubrioperculatus*	CL	South Africa	DQ885024
TZMSC110-05	*L. rubrioperculatus*	CL	South Africa	JF493758
ABFJ011-06	*L. nebulosus*	MH	Japan	JF952783
DSFSG153-10	*L. nebulosus*	MH	Mozambique	HQ561492
FOAC135-05	*L. nebulosus*	MH	Australia	DQ885101
FOAC136-05	*L. nebulosus*	MH	Australia	DQ885102
TZMSB187-04	*L. nebulosus*	MH	South Africa	DQ885020
NNPF120-10	*L. nebulosus*	MH	Iran	HQ149871
TZMSA155-04	*L. nebulosus*	MH	South Africa	DQ885022
FOAC134-05	*L. nebulosus*	MH	Australia	DQ885098
NNPF108-10	*L. nebulosus*	MH	Iran	HQ149872
DSFSE790-08	*L. nebulosus*	MH	Mozambique	JF493754
FOAC133-05	*L. nebulosus*	MH	Australia	DQ885100
FOAC137-05	*L. nebulosus*	MH	Australia	DQ885099
TZMSC427-05	*L. crocineus*	MH	South Africa	JF493744
TZMSC111-05	*L. crocineus*	MH	South Africa	JF493746
TZMSC112-05	*L. crocineus*	MH	South Africa	JF493747
TZMSC289-05	*L. crocineus*	MH	South Africa	JF493745
TZMSB185-04	*L. crocineus*	MH	South Africa	JF493743
TZMSC253-05	*L. crocineus*	MH	South Africa	JF493748
ANGBF4346-12	*L. ornatus*	MH		HQ676773
FOAC123-05	*L. ornatus*	MH	Australia	EF609390
ABFJ012-06	*L. semicinctus*	CL	Japan	JF952784
MBFA758-07	*L. olivaceus*	CL	French Polynesia	JQ431885
FPFLB466-12	*L. olivaceus*	CL	French Polynesia	KJ968135
FOAC102-05	*L. atkinsoni*	MH	Australia	EF609384
FOAC111-05	*L. laticaudis*	MH	Australia	EF609385
CFCS121-08	*L. lentjan*	MH	China	FJ237800
CFCS218-08	*L. lentjan*	MH	China	FJ237799
CFCS219-08	*L. lentjan*	MH	China	FJ237798
CFCS120-08	*L. lentjan*	MH	China	FJ237801
FSCS281-06	*L. lentjan*	MH	China	EF607439
FOAC112-05	*L. lentjan*	MH	Australia	EF609386

Table 1: Continue

BOLD-system ID	Species	Trophic status**	Country	GenBank accession
CFCS119-08	*L. lentjan*	MH	China	FJ237802
FSCS280-06	*L. lentjan*	MH	China	EF607440
GBGC4488-08	*L. miniatus*	CH		EU148533
GBGC4487-08	*L. miniatus*	CH		EU148534
WLIND488-07	*L. miniatus*	CH	India	
WLIND492-07	*L. miniatus*	CH	India	
GBGC4489-08	*L. miniatus*	CH		EU148532
WLIND489-07	*L. miniatus*	CH	India	
DSFSF051-09	*L. mahsena*	MH	Mozambique	JF493751
FOAD529-05	*L. mahsena*	MH	India	EF609387
SBF367-11	*L. mahsena*	MH	Madagascar	JQ350089
DSFSF061-09	*L. mahsena*	MH	Mozambique	JF493752
SBF368-11	*L. mahsena*	MH	Madagascar	JQ350088
DSFSF079-09	*L. mahsena*	MH	Mozambique	JF493750
DSFSG620-11	*L. harak*	MH	Mozambique	KF489626
DSFSG501-11	*L. harak*	MH	South Africa	KF489627
SBF007-11	*L. harak*	MH	Madagascar	JQ350085
SBF097-11	*L. harak*	MH	Madagascar	JQ350086
DSFSG133-10	*L. harak*	MH	Mozambique	HQ561476
ABFJ002-06	*L. harak*	MH	Japan	JF952781
SBF302-11	*L. harak*	MH	Madagascar	JQ350084
GBGC4935-08	*L. obsoletus*	MH		NC_009855
GBGC3728-07	*L. obsoletus*	MH		AP009165
DSFSF040-09	L. sp.	CL	Mozambique	JF493763
DSFSF590-09	L. sp.	CL	Mozambique	JF493762
DSFSF587-09	L. sp.	CL	Mozambique	JF493759
DSFSF589-09	L. sp.	CL	Mozambique	JF493761
DSFSF588-09	L. sp.	CL	Mozambique	JF493760
FPFLB465-12	*L. xanthochilus*	CL	French Polynesia	KJ968136
CFCS027-08	*L. haematopterus*	CH	China	FJ237796
CFCS224-08	*L. haematopterus*	CH	China	FJ237792
CFCS225-08	*L. haematopterus*	CH	China	FJ237791
CFCS221-08	*L. haematopterus*	CH	China	FJ237795
CFCS222-08	*L. haematopterus*	CH	China	FJ237794
CFCS223-08	*L. haematopterus*	CH	China	FJ237793
CFCS026-08	*L. haematopterus*	CH	China	FJ237797
CFCS226-08	*L. haematopterus*	CH	China	FJ237790
DSFSF098-09	*L. microdon*	CL	Mozambique	JF493749
FOAC124-05	*L. ravus*	CL	Australia	EF609391
GBGC4486-08	*L. conchyliatus*	CL		EU148535
WLIND494-07	*L. conchyliatus*	CL	India	
GBGC4485-08	*L. conchyliatus*	CL		EU148536
WLIND493-07	*L. conchyliatus*	CL	India	

**Dentition abbreviation, C: Conical, M: Molariform and submolariform, body types, H: High-bodied, L: Low-bodied

haplotype diversity, nucleotide diversity and average nucleotide difference (K). Source of variation parameters and statistics (FST, FSC and FCT) of analysis of molecular variance (AMOVA) approach were estimated by Arlequin 3.5. The default parameters of these programs were considered and applied.

Before subsequent phylogenetic analyses, BOLD-released COI-sequences of *Lethrinus* species were filtered using some of these softwares to exclude the irrelevant ones which led to paraphyla in this genus on the bases of sequences identity matrix 0.90 within each species using BioEdit 7.1.9. The status of these irrelevant sequences was verified.

For phylogenetic analyses and evolutionary history, *Lethrinus* species COI-sequences set (77 sequences of 18 species) are considered with each of three outgroups (*Gymocranius*, *Gymnocranius*+*Acanthopagrus* and *Gymnocranius*+*Acanthopagrus*+*Acanthurus*+*Lophius*) using three statistical methods: NJ, Neighbor Joining (Kimura 2-parameter method, Kimura, 1980); ML, Maximum Likelihood (Hasegama-kishino Yano/ Gamma distributed with invariant sites, G+I, Nearest-Neighbor-Interchange, NNI) and MP, Maximum Parsimony (Subtree-Pruning-Regrafting, SPR) with bootstrap replication 1000, 500 and 500 respectively in the units of the number of base substitutions per site. Codon

Table 2: BOLD-system ID, GenBank_Accession ID and collection locality for released DNA barcodes COI-sequences of different species used as outgroups in the present study

BOLD-system ID	Species	Country	GenBank accession
DSFSE195-07	*Gymnocranius griseus*	South Africa	JF493569
DSFSE196-07	*Gymnocranius griseus*	South Africa	JF493566
DSFSE197-07	*Gymnocranius griseus*	South Africa	JF493567
DSFSE318-07	*Gymnocranius griseus*	South Africa	JF493568
DSFSE388-08	*Gymnocranius griseus*	South Africa	JF493565
TZMSC109-05	*Gymnocranius* sp.	South Africa	JF493563
ANGBF5347-12	*Acanthopagrus latus*	China	JN242741
ANGBF5348-12	*Acanthopagrus latus*	China	JN242739
ANGBF5424-12	*Acanthopagrus schlegelii schlegelii*	China	JN242575
ANGBF5425-12	*Acanthopagrus schlegelii schlegelii*	China	JN242573
ANGBF5483-12	*Acanthopagrus latus*	China	JN242740
ANGBF5484-12	*Acanthopagrus latus*	China	JN242738
ANGBF5560-12	*Acanthopagrus schlegelii schlegelii*	China	JN242574
ANGBF5561-12	*Acanthopagrus schlegelii schlegelii*	China	JN242572
ANGEN130-15	*Acanthopagrus latus*	India	
DSFSE001-07	*Acanthopagrus vagus*	South Africa	JF492775
DSFSE539-08	*Acanthopagrus berda*	South Africa	JF492773
FOA660-04	*Acanthopagrus australis*	Australia	DQ107852
FOA661-04	*Acanthopagrus australis*	Australia	DQ107853
FOA662-04	*Acanthopagrus australis*	Australia	DQ107854
FOA663-04	*Acanthopagrus australis*	Australia	DQ107855
FOA664-04	*Acanthopagrus australis*	Australia	DQ107856
FOA665-04	*Acanthopagrus berda*	Australia	DQ107857
FOA667-04	*Acanthopagrus berda*	Australia	DQ107844
FOA668-04	*Acanthopagrus berda*	Australia	DQ107845
FOA669-04	*Acanthopagrus berda*	Australia	DQ107846
ANGBF5988-12	*Acanthurus mata*		FJ459543
ANGBF6078-12	*Acanthurus mata*		FJ459542
ANGBF6615-12	*Acanthurus triostegus*		JF718092
ANGBF6616-12	*Acanthurus triostegus*		JF718090
GBGC8826-09	*Lophius americanus*	Canada	EU683979
GBGC8827-09	*Lophius americanus*	Canada	EU683978
GBGC8828-09	*Lophius americanus*	Canada	EU683977
GBGC8829-09	*Lophius americanus*	Canada	EU683976

positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset.

RESULTS

Sequence analysis: About 98 COI-sequences representing 18 *Lethrinus* species were considered in the current species released from BOLD SYSTEM database. These sequences representing different geographical regions including China, Australia, Japan, Taiwan, India, Iran, Madagascar, Mozambique and South Africa. The COI-sequences extended from 174-1548 bp with an average of 660±11.6. However, without extremes recorded in *L. nebulosus* and *L. obsoletus*, the other 94 COI-sequences representing the 18 species ranged from 590-682 with a mean of 649±1.64 bp. The NJ-based phylogenetic tree (Fig. 1) of all sequences (only 121 positions included in the final data set) exhibited paraphyletic and monophyletic species. The latter monophyletic groups include *L. obsoletus*, *L. crocineus*, *L. harak* and *L. haematopterus*. The remaining species are paraphyletics (Fig. 1). After, excluding the minicode sequence of 174 bp of *L. nebulosus* (NNPF178-10), the same situation of species paraphyla and monophyla with some variations is still recorded in spite of increased number of positions included in the final data set (527 bp) using different statistical methods (NJ, ML and MP). Accordingly, the within species variations and identity matrix in each *Lethrinus* species were examined by BioEdit program and in within each species sequences having identity less than 0.90 were excluded from subsequence analyses in an attempt to resolve the phylogenetic trees and the interspecific relationships.

Lethrinus nebulosus COI-sequences exhibited intraspecific variations including geographic ones. After exclusion of 5 sequences of *L. nebulosus* with Id<0.90, the overall mean distance (K2P) decreased from 0.185±0.014 for 16 sequences to 0.05±0.004 for sequences with Id 0.93. Moreover, the variable sites and parsimony informative sites

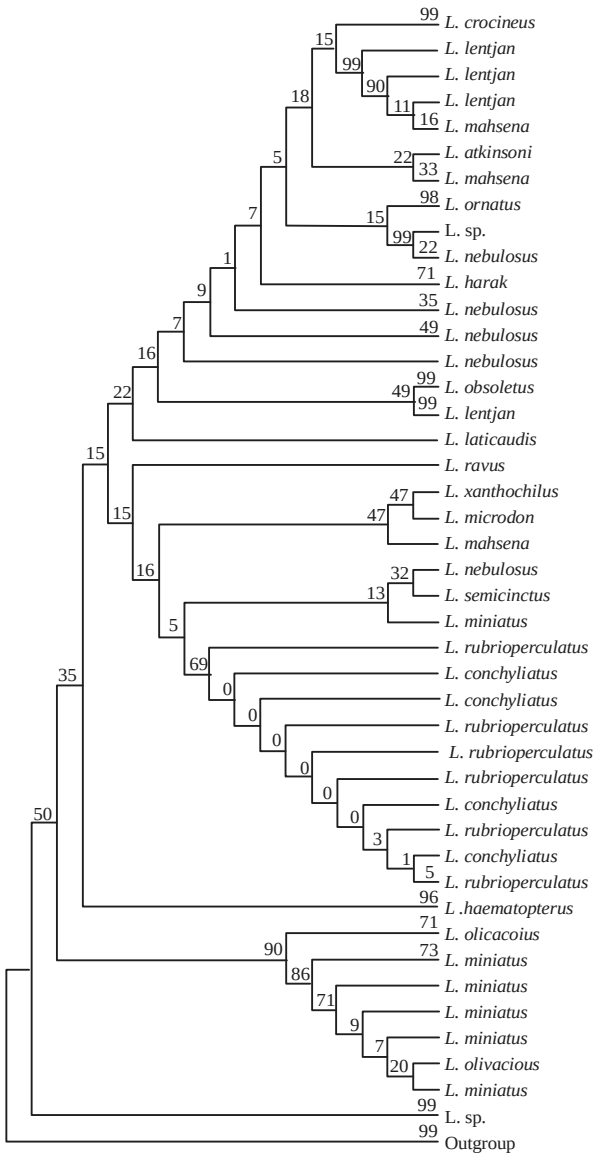

Fig. 1: Phylogenetic tree (based on the Neighbour Joining; Kimura-2 parameter) of the BOLD-system released 97 COI-sequences of 18 *Lethrinus* species with 6 COI-sequences of *Gymnocranius* species as outgroup (121 nucleotide positions are included in final data set, 1000 replications, bootstrapped)

reduced from 344-44 and from 57-40, respectively. These sequences referred to two lineages: Japan-Australia and Mozambique-Iran; South Africa sequences appeared as sister group to these two lineages (Fig. 2a). However, the parsimony phylogenetic tree of all 16 sequences identifies the indian sequences as ancestor of the other sequences which reflects two different directions of *L. nebulosus* spreading (Fig. 2b): One towards the east (Australia-Japan-Taiwan) and the other towards the west (Iran-Mozambique-South Africa). However, in the subsequent PM and ML-based phylogenetic analyses of different final sets of *Lethrinus* species in concern, the two South African sequences of *L. nebulosus* (TZMSA155-04 and TZMSB187-04) were clustered as sister group to *L. ornatus*. So, inspite of their ID higher than 0.9, the specimen identification of these sequences as *L. nebulosus* is questionable.

Intraspecific variations of *L. lentjan* were also evident since its 11 COI-sequences are classified into two main lineages: China-Australia and Iran (Fig. 2c). Iran sequences led to paraphyla in subsequent analyses. So, these sequences (Id<0.90) are excluded from the subsequent analyses of *Lethrinus* species. Accordingly, the overall mean distance decreased from 0.073±0.007 to 0.005±0.002. Moreover, the

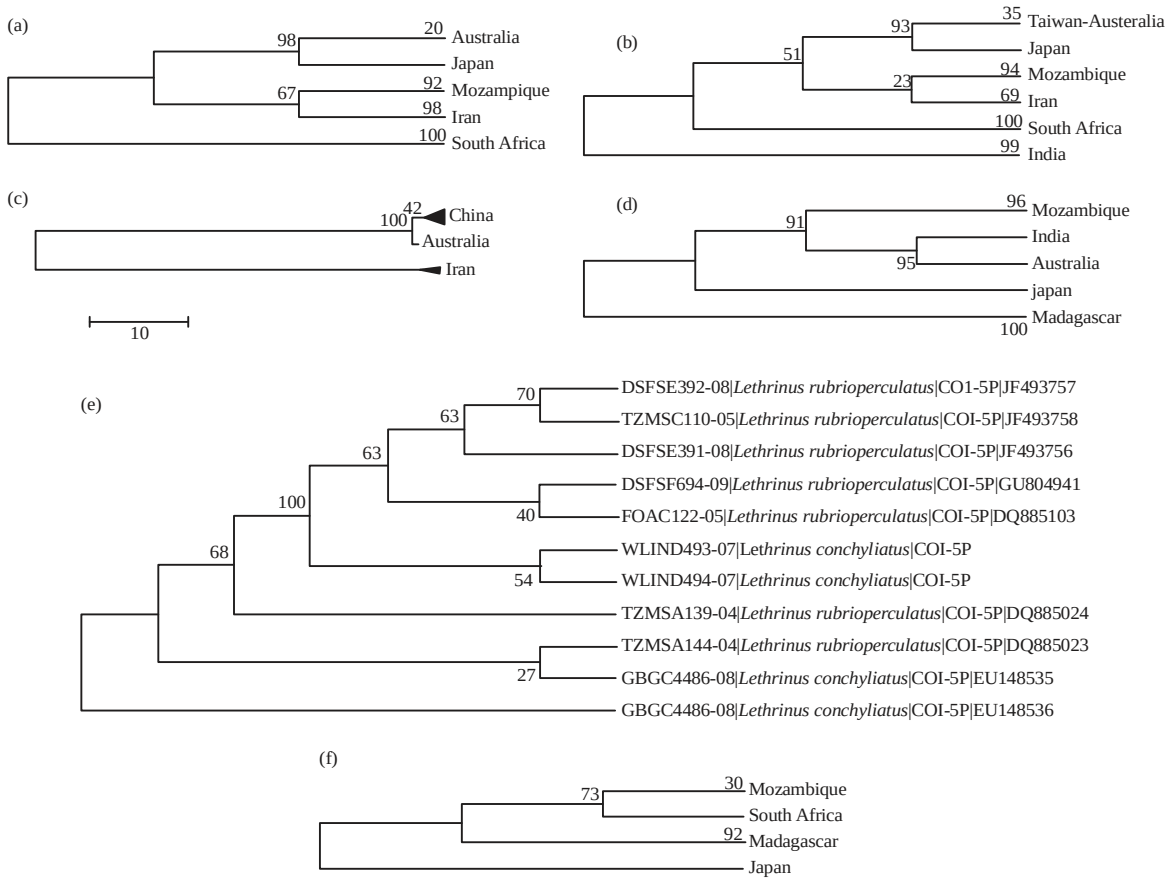

Fig. 2(a-f): Maximum parsimony-based phylogenetic trees (1000 replications) of the BOLD-system released COI-sequences of some *Lethrinus* species showing within species variations (a) *L. nebulosus* (only 12 COI sequence with ID>0.9, 639 position included in final data set with booting), (b) *L. nebulosus* (all 16 COI sequence; 534 position included in final data set with booting), (c) *L. lentjan* (11 COI sequences; 641 position included in final data set), (d) *L. mahsena* (8 coi sequences; 651 position included in final data set), (e) *L. rubrioperculatus* and *L. conchyliatus* (552 position included in final data set) and (f) *L. harak* (7 COI sequences; 650 position included in final data set)

polymorphic sites changed from 87-8 with high reductions in other parameters such as haplotype and nucleotide diversities and average number of nucleotide differences.

Lethrinus atkinsoni sequence (FOAC 102) of BOLD-system 98 released COI-sequences is included with *L. mahsena* sequence set since all preliminary analyses of its phylogenetics emphasized on its affiliation to *L. mahsena* not to *L. atkinsoni* (Fig. 2d). After the removal of Japanese sequences (ABFJ022-06) of *L. mahsena* with Id<0.90, the overall mean distance slightly reduced from 0.083±0.008 to 0.057±0.007 with variations in other parameters. *Lethrinus mahsena* sequence set exhibited geographic variations in Mozambique, Madagascar, India, Australia and Japan regions. The excluded Japanese sequence is clustered with Madagascar sequences in the booted phylogenetic parsimony tree.

One of *L. rubrioperculatus* sequences (TZMSC144) with Id>0.90 is excluded. So, the overall mean distance reduced from 0.863±0.118 to 0.002±0.001. However, on clustering, these sequences interfere in the subsequent analyses only with the *L. conchyliatus* sequences in spite of their Id matrix 0.94 up to 1 (Fig. 2e). Accordingly, other three *L. rubrioperculatus* sequences (DSF391, DSF694 and FOAC122) are excluded to prevent paraphyla in subsequent analyses.

The FOAC117-05 sequence with Id<0.90 is excluded from *L. miniatus* set leading to little change in the overall mean distance from 0.053±0.005 to 0.002±0.001. Six sequence set of *L. miniatus* (Id>0.99) separated as a single unite in all subsequent analyses. *Lethrinus olivaceus* sequences leading to paraphyla in *L. miniatus* and *L. olivaceus* complex are also excluded in final analyses of *Lethrinus* species. The DSFSE398 and FOAC126 sequences of *L. olivaceus* may belong to *L. miniatus* since these sequences are separated within *L. miniatus* set.

Except for DSFSE390 sequence with Id<0.90, all L. sp. sequences (Id>0.99) are separated as one unite in all subsequent phylogenetic analyses by different methods. Also, each of the sequence sets of *L. concineus*, *L. harak*, *L. haematopterus* and *L. obsoletus* is separated as one unit with no paraphyla under different methods; their Id matrices have very high values. The majority of these species exhibited intraspecific variations including geographic ones. In *L. harak* sequence set (Id>0.95 up to 0.998), there are two lineages: Mozambique-South Africa and Madagascar; Japanese sequence represent an ancestor to these lineages (Fig. 2f). In *L. haematopterus* sequence set, phylogenetics refer to two main groups in the same locality under different statistical methods and analyses. Each of the other six *Lethrinus* species is only represented by one sequence.

According to the aforementioned inspection and analysis of Bold-system released COI-data of *Lethrinus* species, only 77 sequences out of 98 ones are included in the subsequent phylogenetic analyses. Outgroups used in these analyses are represented by some sequences of *Gymnocranius* (Family: Lethrinidae), *Acanthopagrus* (Family: Spariidae, a sister group to Lethrinidae), *Lophius* (Family: Lophiidae) and/or *Acanthurus* (Family: Acanthuridae).

The overall mean distance for 77-sequences set of 18 *Lethrinus* species is 0.140±0.011 whereas these distances with *Gymocranius* outgroup and *Lethrinus*-all outgroups set are 0.149±0.011 and 0.182±0.011, respectively. The within mean group distance of *Lethrinus*-all outgroups set is 0.009±0.003 with range of 0.00-0.059, nearly the same for *Lethrinus* species only (0.009±0.005). The between group mean distance of *Lethrinus* species is 0.153±0.003 (0.005-0.214) whereas those between *Lethrinus* species and each of *Gymnocranius*, *Acanthopagrus*, *Acanthurus* and *Lophius* outgroups are 0.211±0.002 (0.193-0.231), 0.230±0.002 (0.186-0.262), 0.228±0.003 (0.143-0.259) and 0.227±0.003 (0.206-0.254). The total between group mean distance for *Lethrinus*-all outgroup set is 0.193±0.003 (0.005-0.266).

For phylogenetic analyses, *Lethrinus* species COI-sequences set (77 sequences of 18 species) are considered with each of three outgroups (*Gymocranius*, *Gymnocranius*+*Acanthopagrus* and *Gymnocranius*+*Acanthopagrus*+*Acanthurus*+*Lophius*) using three statistical methods: NJ (K2P), ML (Hasegama-kishino Yano/G+I) and MP (Subtree-Pruning-Regrafting) with bootstrap replication of 1000, 500 and 500, respectively. The population parameters of these four sets used are given in Table 3. In all cases, there are two lineages of *Lethrinus* species (Fig. 3-5). The first lineage (Lineage 1) includes *L. lentjan*, *L. obsoletus*, *L. ornatus*, *L. crocineus*, *L. nebulosus*, *L. harak*, *L. mahsena* and *L. laticaudis* and the second one (Lineage 2) comprises *L. semicinctus*, *L. ravus*, *L. conchyliatus*, *L. rubrioperculatus*, *L. microdon*, *L. xanthochilus*, L. sp., *L. olivaceus*, *L. miniatus* and *L. haematopterus* acts as a sister group to the two lineages in the majority of cases of the three statistical methods used for the three sets of data with different outgroups. The minimum evolution tree not mentioned here, also emphasized on such conclusion. However, in the booted PM of 83-set and NJ of 103-set, *L. haematopterus* act as a sister group to lineage 1. Also, this species act as a sister group to lineage 2 only by unbooted MP analysis of 103-set. As a results, one can postulated *L. haematopterus* as an ancestor to the two COI-based lineages of *Lethrinus* recorded.

Lethrinus conchyliatus and *L. rubrioperculatus* sequences are clustered together in all cases. The following groupings of species are recorded to be constant in all analyses with different methods: *L. lentjan*+*L. obsoletus*, *L. harak*+*L. mahsena*, L. sp.+*L. olivaceus*+*L. miniatus*, *L. semicinctus*+*L. ravus* and *L. microdon*+*L. xanthochilus*. These interspecific genetic relationships reflect in the majority of cases, the corresponding morphological ones in taxonomic case. Except for NJ analyses of 83-set and 103-set, *L. nebulosus* sequences are distributed between two groups in all cases, one represents the majority of its sequences and the other one

Table 3: Some population parameters of the four COI-sets used in the final COI-based phylogenetic analyses of 18 *Lethrinus* species in concern

Parameters	77-*Lethrinus* set	83-*Lethrinus*-outgroup1 set*	103-*Lethrinus*-outgroup2 set*	111-*Lethrinus*-outgroup3 set*
No of polymorphic sites	203	209	223	209
G+C	0.493	0.493	0.483	0.478
No. of mutations	302	334	374	575
No. of haplotypes	46	48	58	62
Haplotype diversity	0.98200±0.005	0.98100±0.005	0.98500±0.003	0.98500±0.003
Nucleotide diversity	0.12252±0.004	0.13003±0.004	0.14710±0.0036	0.15622±0.004
No. of parsimony informative sites	199	205	219	207
Average nucleotide difference (K)	73.5	78.01	88.26	84.2
Total sites included	600	600	600	539
Sequence conservation	0.660	0.650	0.524	0.604
Conservative threshold	0.75	0.75	0.72	0.70

*Outgroup 1: *Gymocranius*, outgroup 2: *Gymnocranius*+*Acanthopagrus* and outgroup 3: *Gymnocranius*+*Acanthopagrus*+*Acanthurus*+*Lophius*

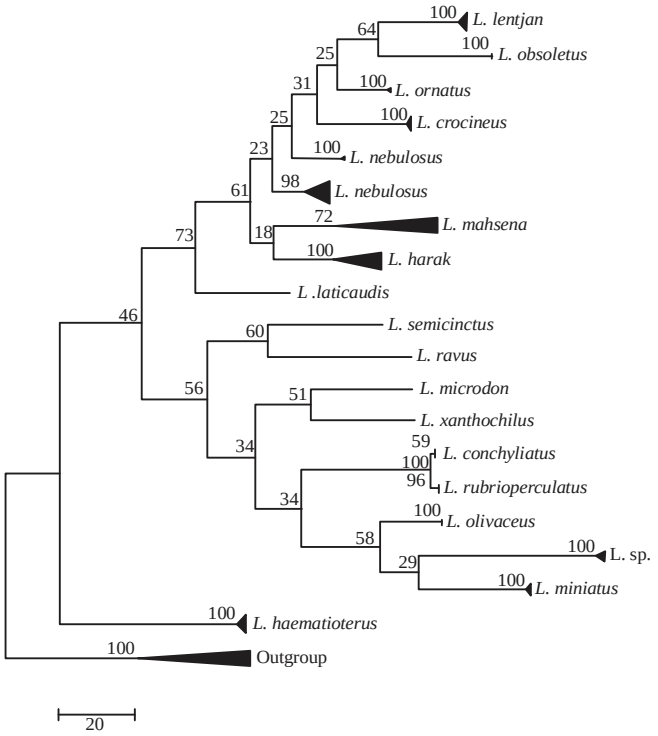

Fig. 3: Maximum parsimony-based phylogenetic tree of the BOLD-system released 77 COI-sequences of 18 *Lethrinus* species with 6 COI-sequences of *Gymnocranius* species as outgroup (600 nucleotide positions are included in final data set, 500 replications)

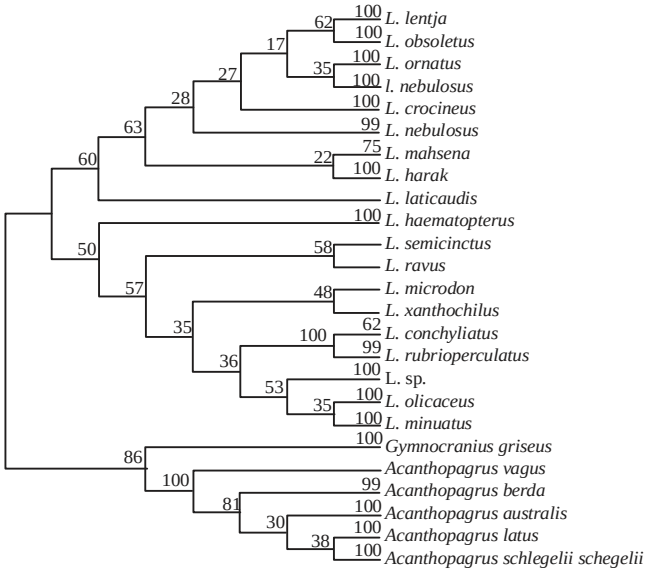

Fig. 4: Maximum parsimony-based phylogenetic tree of the BOLD-system released 77 COI-sequences of 18 *Lethrinus* species with 26 COI-sequences of *Gymnocranius* (6) and *Acanthopagrus* (20) species as outgroup (600 nucleotide positions are included in final data set, 500 replication, bootstrapped)

includes only the two South African sequences which are clustered as sister group to *L. ornatus*. In lineage 1, *L. laticaudis* is found to be ancestor to the remaining species in the majority of cases. However, in NJ and booted ML of 111-set, *L. mahsena* resolves as sister to lineage 1. These latter rare cases may be due to inclusion of the outgroup species

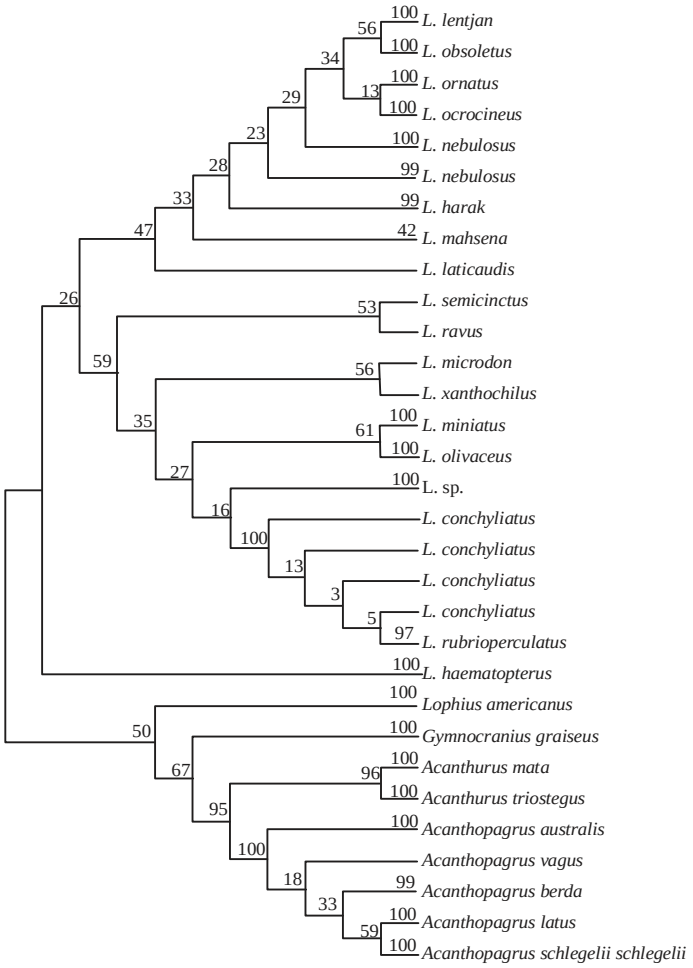

Fig. 5: Maximum parsimony-based phylogenetic tree of the BOLD-system released 77 COI-sequences of 18 *Lethrinus* species with 34 COI-sequences of *Gymnocranius* (6), *Acanthopagrus* (20), *Acanthurius* (4) and *Lophius* (4) species as outgroup (539 nucleotide positions are included in final data set, 500 replication)

away of Lethrinidae and Sparidae (the lethrinid sister group) and the corresponding bootstrapping.

Haplotype and AMOVA analyses: Out of 600 nucleotide positions of 83-set included in the analysis, 209 are found to be variable with 205 parsimony informative sites, G+C of 0.443, mutations of 334, haplotypes of 48, high haplotype diversity of 0.981±0.005, nucleotide diversity of 0.13003±0.004 and average nucleotide difference of 78.01. The average nucleotide compositions of the 83-set species are given in Table 4. The distribution of the 48 haplotypes (Fig. 6, 7) between species is given in Table 5; no sharing in haplotypes between species is recorded. The tree of these haplotypes on statistical bases of NJ and MP reflects a pattern of haplotype grouping corresponding to that of tree-based sequences since two lineages were identified with haplotypes of *L. haematopterus* as sister group to the two lineages; haplotypes of *Gymnocranius* sequences were found to be a sister group to *Lethrinus* species considered. The genetic distance (K2P) between haplotypes ranged between 0.005-1.52 with an average of 0.600±0.009. Also, p-distance averaged 0.347±0.004 with a range of 0.005-0.563.

As reflect by phylogenetic analyses, the 83-sequence set of the 18 *Lethrinus* species considered with the outgroup, *Gymnocranius* species are classified into four groups (clades) for further analysis by AMOVA (Fig. 3, Table 6). These groups include sequences of species of lineage 1 (L1), species of lineage 2 (L2), *L. haematopterus* (LH) and the outgroup. The between group average distances (K2P) of the first *Lethrinus* three groups (L1 and L2 = 0.117±0.014, L1 and LH = 0.177±0.016 and L2 and LH = 0.186±0.016) were greater than their within group average distances (L1, L2 and LH = 0.087±0.007, 0.121±0.010 and 0.004±0.002, respectively). The AMOVA statistics (FST, FSC and FCT) are

Table 4: Average nucleotide compositions of COI-sequences of *Lethrinus* species and outgroup in concern

Species	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
L. rubrioperculatus	28.9	28.9	23.3	18.9	16.3	26.9	25.7	31.1	42.2	28.4	15.0	14.4	28.3	31.4	29.1	11.1
L. conchyliatus	28.6	29.1	23.3	19.1	16.3	27.1	25.3	31.3	41.8	28.0	15.2	14.9	27.5	32.1	29.4	11.0
L. sp.	26.9	30.0	24.5	18.5	16.6	26.7	25.4	31.4	41.9	28.1	15.2	14.7	22.3	35.3	32.8	9.5
L. mahsena	27.0	29.8	23.2	20.0	16.7	26.7	25.4	31.3	42.0	28.2	15.2	14.7	22.4	34.6	29.1	13.9
L. ornatus	27.7	29.9	21.8	20.6	16.8	26.7	25.3	31.3	42.1	28.0	15.2	14.7	24.3	34.9	25.0	15.8
L. olivaceus	27.3	29.9	23.1	19.8	16.8	26.7	24.8	31.7	42.1	28.0	15.2	14.7	22.9	34.9	29.4	12.8
L. nebulosus	28.2	29.1	22.9	19.7	16.6	26.7	25.4	31.3	42.0	28.2	15.1	14.8	26.1	32.5	28.2	13.2
L. miniatus	28.7	27.6	22.9	20.8	17.7	25.7	24.8	31.7	41.8	28.3	15.2	14.7	26.5	28.9	28.7	15.9
L. lentjan	27.4	29.8	22.5	20.3	16.6	26.8	25.3	31.3	42.0	28.1	15.2	14.7	23.7	34.6	26.9	14.7
L. harak	27.1	30.1	22.2	20.6	16.6	26.7	25.5	31.3	41.9	28.3	15.2	14.7	22.8	35.5	26.0	15.7
L. haematopterus	27.1	31.2	20.6	21.1	16.1	27.2	25.3	31.3	41.9	28.1	15.2	14.7	23.3	38.2	21.2	17.3
L. crocineus	27.9	29.9	21.8	20.4	16.3	26.9	25.7	31.2	42.3	28.3	14.9	14.5	25.1	34.4	25.0	15.5
L. semicinctus	29.1	28.5	23.2	19.2	15.7	27.6	25.3	31.3	41.9	28.1	15.2	14.7	29.8	29.8	28.9	11.5
L. microdon	29.2	28.9	22.1	19.8	17.5	25.8	25.3	31.3	41.9	28.1	15.2	14.7	28.1	32.7	25.8	13.4
L. laticaudis	28.7	29.2	22.7	19.4	17.4	26.1	25.2	31.2	42.2	28.0	15.1	14.7	26.5	33.3	27.9	12.3
L. ravus	27.9	29.6	23.4	19.1	16.5	27.1	25.2	31.2	42.2	28.0	15.1	14.7	25.1	33.8	29.7	11.4
L. xanthochilus	29.6	28.5	22.1	19.7	16.5	27.1	25.2	31.2	42.2	28.0	15.1	14.7	30.1	30.6	26.0	13.2
L. obsoletus	28.8	28.8	22.6	19.8	18.3	24.0	26.6	31.1	41.3	27.2	16.2	15.4	26.8	35.2	25.0	13.0
Outgroup (*Gymnocranius*)	27.6	31.6	23.0	17.8	16.5	27.2	26.3	30.0	41.9	28.6	15.2	14.4	24.4	39.1	27.4	9.1

Table 5: Distribution of the 48 COI-based haplotypes among *Lethrinus* species and outgroup in concern

Haplotype	*L. rubrioperculatus*	*L. conchyliatus*	L. sp.	*L. mahsena*	*L. ornatus*	*L. olivaceus*	*L. nebulosus*	*L. miniatus*	*L. lentjan*	*L. harak*	*L. haematopterus*	*L. crocineus*	*L. semicinctus*	*L. microdon*	*L. laticaudis*	*L. ravus*	*L. xanthochilus*	*L. obsoletus*	Outgroup	Total
Hap_1:	3																			3
Hap_2:		4																		4
Hap_3:			3																	3
Hap_4:			1																	1
Hap_5:			1																	1
Hap_6:				2																2
Hap_7:				1																1
Hap_8:				1																1
Hap_9:				1																1
Hap_10:				1																1
Hap_11:				1																1
Hap_12:					1															1
Hap_13:					1															1
Hap_14:						2														2
Hap_15:							4													4
Hap_16:							1													1
Hap_17:							1													1
Hap_18:							1													1
Hap_19:							1													1

Table 5: Continue

Haplotype	*L. rubrioperculatus*	*L. conchyliatus*	L. sp.	*L. mahsena*	*L. ornatus*	*L. olivaceus*	*L. nebulosus*	*L. miniatus*	*L. lentjan*	*L. harak*	*L. haematopterus*	*L. crocineus*	*L. semicinctus*	*L. microdon*	*L. laticaudis*	*L. ravus*	*L. xanthochilus*	*L. obsoletus*	Outgroup	Total
Hap_20:							1													1
Hap_21:							1													1
Hap_22:							1													1
Hap_23:							1													1
Hap_24:								4												4
Hap_25:								2												2
Hap_26:									3											3
Hap_27:									1											1
Hap_28:									1											1
Hap_29:									1											1
Hap_30:									1											1
Hap_31:									1											1
Hap_32:										1										1
Hap_33:										3										3
Hap_34:										2										2
Hap_35:										1										1
Hap_36:											3									3
Hap_37:											5									5
Hap_38:												4								4
Hap_39:												1								1
Hap_40:												1								1
Hap_41:													1							1
Hap_42:														1						1
Hap_43:															1					1
Hap_44:																1				1
Hap_45:																	1			1
Hap_46:																			5	5
Hap_47:																			1	1
Hap_48:																		2		2
Total	3	4	5	7	2	2	12	6	8	7	8	6	1	1	1	1	1	2	6	83

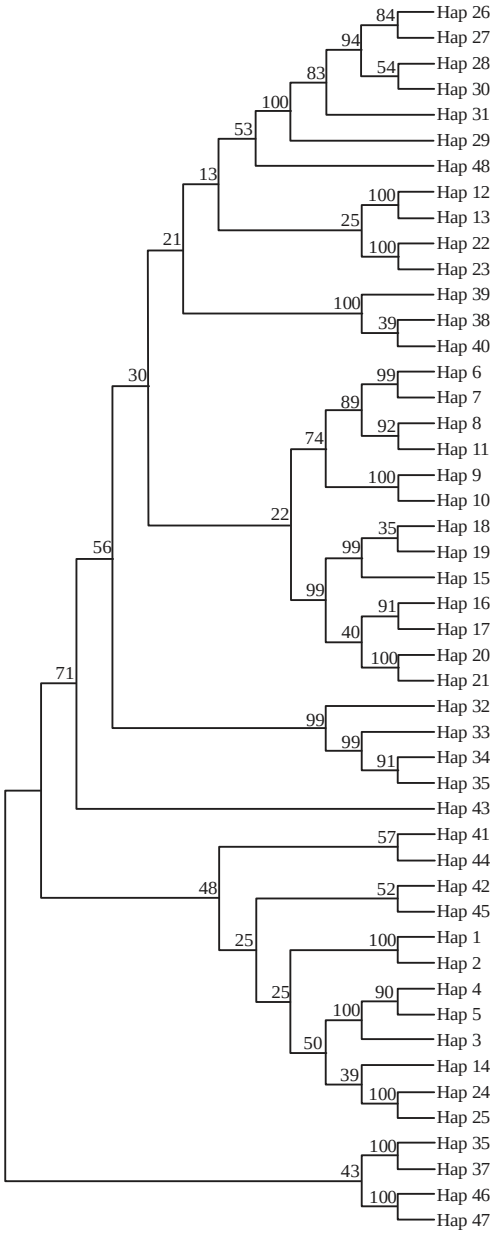

Fig. 6: Maximum parsimony-based phylogenetic tree of the 48 haplotypes of the BOLD-system released 77 COI-sequences of 18 *Lethrinus* species with 6 COI-sequences of *Gymnocranius* species (208 nucleotide positions are included in final data set, 1000 replications)

Table 6: AMOVA analysis of the four groups (Lineage 1, lineage 2, *L. haematopterus* and the outgroup, *Gaymncranius* species) of the 83-COI sequence set of *Lethrinus* species studied

Source of variation	DF	Sum of squares	Variance component	Variation (%)
Among groups	3	5.321	0.02533 Va	4.95
Among species within groups	15	14.056	0.16006 Vb	31.31
Within species	64	20.852	0.32582 Vc	63.74
Total	82	40.229	0.5112	
Fixation indices				
FCT_Va	0.04954		0.02835	
FSC_Vb	0.32942	p =	0.00000	
FST_Vc	0.36264		0.00000	
Permutations	1023			

significant with p>0.00000, 0.00000 and 0.02835, respectively (Table 6). These findings referrers to significance between the four clades identified in 83-sequence set as well as between species within lineages. Overall average FST value referred to great genetic differentiation among species. Individual FST matrix between species at significance of 0.05 is given in Table 7. Also, the variability in genetic structure characteristics of the four clades or groups is given in Table 8.

The two COI-based lineages identified in current *Lethrinus* species phylogenetics are found to be correlated with the two dentition-body patterns (HM and LC) with no overlapping except for *L. miniatus* (a mesocarnivore generalist, HC which is clustered with *L. olivaceus* (CL) and L. sp. (CL) as subclade in lineage 2 (the mesocarnivore stalkers, CL) in all analyses based on different statistical methods. *L. haematopterus* (HC) in the majority of cases as postulated previously resolves as the most ancestral species and

Table 7: Computing conventional *Lethrinus* pairwise FSTs matrix from COI-haplotype frequencies and matrix of significance values in AMOVA at 0.05 level, permutations = 110

Species**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
L_rub		0.034	0.071	0.078	0.119	0.217	0.048	0.288	0.163	0.424	0.371	0.248	0.048	0.048	0.048	0.048	0.288	0.263	0.342
L_conc	-		0.067	0.078	0.138	0.294	0.000	0.500	0.212	0.724	0.647	0.347	0.000	0.000	0.000	0.000	0.500	0.357	0.510
L_SP	-	-		0.099	0.136	0.222	0.091	0.290	0.176	0.394	0.355	0.249	0.091	0.091	0.091	0.091	0.290	0.261	0.326
L.mah	+	-	+		0.148	0.243	0.107	0.319	0.192	0.441	0.393	0.273	0.107	0.107	0.107	0.107	0.319	0.286	0.362
L_orn	+	-	+	+		0.291	0.190	0.383	0.240	0.503	0.456	0.322	0.190	0.190	0.190	0.190	0.383	0.333	0.416
L_oli	+	-	+	+	+		0.400	0.538	0.353	0.642	0.600	0.433	0.400	0.400	0.400	0.400	0.538	0.435	0.533
L_neb	-	-	-	-	-	-		1.000	0.300	1.000	1.000	0.467	1.000	1.000	1.000	1.000	1.000	0.464	0.667
L_min	-	-	+	-	+	+	-		0.483	1.000	1.000	0.586	1.000	1.000	1.000	1.000	1.000	0.569	0.735
L_lent	+	-	+	+	+	+	-	+		0.612	0.561	0.389	0.300	0.300	0.300	0.300	0.483	0.395	0.497
L_har	+	-	+	+	+	+	-	-	+		1.000	0.680	1.000	1.000	1.000	1.000	1.000	0.650	0.797
L_haem	+	-	+	+	+	+	-	-	+	+		0.642	1.000	1.000	1.000	1.000	1.000	0.617	0.771
L_cro	+	-	+	+	+	+	-	+	+	+	+		0.467	0.467	0.467	0.467	0.586	0.465	0.567
L_sem	-	-	-	-	-	-	-	-	-	-	-	-		1.000	1.000	1.000	1.000	0.464	0.667
L_mic	-	-	-	-	-	-	-	-	-	-	-	-	-		1.000	1.000	1.000	0.464	0.667
L_lat	-	-	-	-	-	-	-	-	-	-	-	-	-	-		1.000	1.000	0.464	0.667
L_rav	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		1.000	0.464	0.667
L_xan	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-		0.569	0.735
outg	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+		0.554
L_obs	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+	+	

**Species abbreviations include the first three or four letters of the species name, outg: Outgroup, *Gymnocranius* species

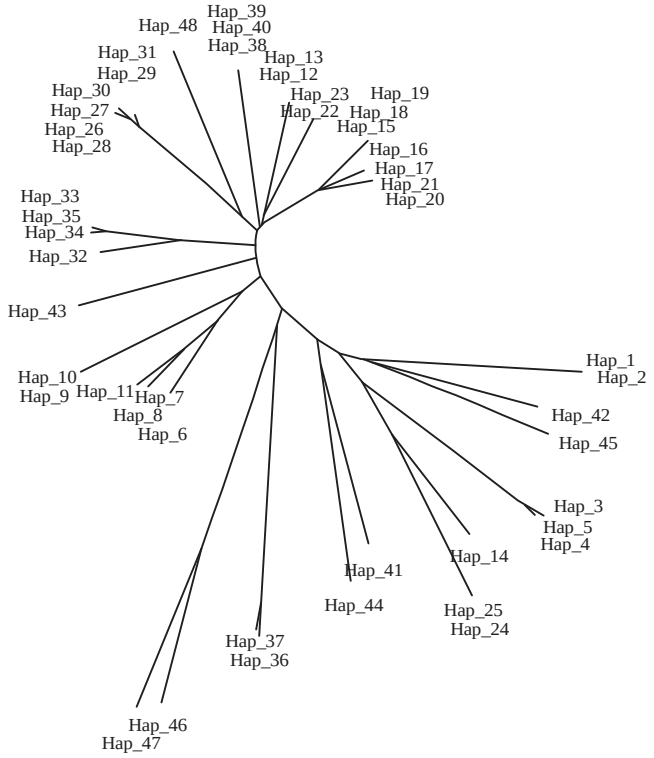

Fig. 7: Splitstree phylogenetic tree of the 48 haplotypes of the BOLD-system released 77 COI-sequences of 18 *Lethrinus* species with 6 COI-sequences of *Gymnocranius* species

Table 8: Genetic structural characteristics of the four clades or groups identified on the bases of phylogenetic analyses of the 83-COI-set of the 18 *Lethrinus* species in concern with *Gymnocranius* sequences as outgroup

Parametrics	Lineage 1	Lineage 2	*L. haematopeculus*	Outgroup
Sample size	45	24	8	6
No. of haplotypes	32	12	2	2
Deletion weight	1	1	1	1
Transition weight	1	1	1	1
Transversion weight	1	1	1	1
Allowed level of missing data (%)	5	5	5	5
No. of transitions	164	185	4	52
No. of transversions	37	55	0	8
No. of substitutions	201	240	4	60
No. of indels	378	5	0	0
No. of ts. sites	157	156	4	52
No. of tv. sites	37	55	0	8
No. of subst. sites	170	185	4	60
No. of private subst. sites	24	38	0	3
No. of indel sites	378	5	0	0
Pi	50.72	70.13	2.14	20.00
No. of polymorphic sites	537	189	4	60
Theta_k	48.06	8.88	0.49	0.59
Theta_k_lower	26.26	4.09	0.11	0.13
Theta_k_upper	90.05	19.14	2.08	2.63
Theta_H	44.31	11.34	0.86	0.37
s.d. Theta_H	22.3	5.01	0.43	0.36
Theta_S	38.88	49.54	1.54	26.28
s.d. Theta_S	11.24	16.27	0.96	12.66
Theta_pi	83.53	71.59	2.14	20
s.d. Theta_pi	40.65	35.64	1.51	11.94
Nucleotide composition (Relative values) (%)				
C	29.58	28.90	31.17	31.62
T	27.69	28.23	27.11	27.63
A	22.61	23.36	20.59	22.96
G	20.11	19.50	21.13	17.79

positions sister to all remaining *Lethrinus* species. In addition, *L. laticaudis* (HM) positions sister to the remaining species in lineage 1 (mesocarnivore specialist, HM) in the majority of cases of current phylogenetic analyses.

DISCUSSION

The DNA barcoding is a well established method for specimen identification and species discovery using a short standardized region (648 bp, the folmer region) of DNA[24,42,51,52]. Potentially, this regions contain enough information to resolve 10-100 million species[24]. However, misconceptions pervading the DNA barcoding literature were considered and highlighted by Collins and Cruickshank[42] in terms of 7 deadly sins of DNA barcoding with suggestions of possible improvements. Some of these suggestions and those of Lv *et al.*[53] were applied to the analysis of the 97 *Lethrinus* species sequences in concern and about 20 sequences were excluded to prevent paraphyla and to build resolved phylogenetic tree of *Lethrinus* species considered. Some of these sequences represented inadequate a priori identification of specimens and their possible correct identification was suggested according to liberal tree-base method[53]. Accordingly, one can suggest that GenBank and BOLD system database sequences must be accepted for phylogenetic analysis after their filtering and validity analysis to avoid the deadly sins of DNA and misconception and hence transformation of morphology-based taxonomic problems to these databases.

Hajibabaei and McKenna[54] referred to difficulty of getting a full length DNA barcode in older preserved museum specimens and in processed biological materials such as food products, pharmaceuticals and nutraceuticals. So, DNA mini-barcodes have been recovered with effectiveness in biodiversity analysis and in specimen identification to a species level in museum samples[54-56]. In the present study, NJ-based phylogenetic tree of all sequences (only 121 positions included in the final data set due to sequence (174 bp) of *L. nebulosus* specimen (NNPF178-10) exhibited paraphyletic and monophyletic species. After removal of the inadequate sequences except that of NNPF178-10, paraphyla disappeared and the pattern of *Lethrinus* two lineages with the sister group, *L. haematopterus* (postulated later with 600 postion included) was not resolved since 137 positions are included in the final data set. This finding referred to the little information reflected by mini-codes.

Some researchers and investigators applied more than one statistical method and model[2] in phylogenetic studies and others used only one method[57]. These statistical methods are built on different assumptions and produced minor variable outputs especially on the interspecific relationships. Bootstrapping also exhibits such minor variability. These minor variations do not prevent the resolution of phylogenetic trees in sequence data freed of inadequate ones prior to phylogenetic analyses. Such situation was evident in the present study since the outputs of the majority of method and models referred to similar pattern of COI-based *Lethrinus* interspecific relationship.

The three trophic categories, LC, HC and HM recorded in emperor fishes, genus *Lethrinus* in relation to body form and dentition type were morphologically, genetically and evolutionary considered by different researchers[2,3,12]. Carpenter and Allen[1] and Carpenter[12] described the variations in the body-feeding-habitat characteristics of these tropical and sub-tropical fishes which distributed mostly in the Indo-West Pacific. Al-Sufiani[7] referred to the head region of some *Lethrinus* species to be morphologically important in their differention by truss characteristics. Other researchers including Alexander[58] referred to general trends in the evolution of body shape in relation to swimming behavior, habitat and feeding types. However, using morphology in inferring trophic type evolution of *Lethrinus* species is problematic since these morphological characters are typically correlated with feeding mode and are potentially homoplasious[2]. These researchers used mitochondrial DNA sequences (cytochrome b) to determine phylogenetic hypothesis for *Lethrinus*, not related to trophic morphological characteristics. The results of Lo Galbo *et al.*[2] clearly inferred a monophyletic *Lethrinus* with two well defined lineages within *Lethrinus* exhibiting two distinct trophic types (LC and HM)) as delineated by Carpenter[12]. In the present study, similarly these two lineages were well identified on the basis of DNA barcoding of COI with different in their ancestor (sister group). These findings as postulated by Lo Galbo *et al.*[2] indicated the primary radiation with *Lethrinus* occurred separately thin these two lineages. In contrast, the same trophic type evolved separately several times in the more speciose Sparidae[11,13].

Using cytochrome b, Lo Galbo *et al.*[2] recorded *L. miniatus* (HC) as a well-supported *Lethrinus* clade aside from the two primary trophic groups, working as basal species, sister to all other *Lethrinus* species. On the other hand, in the COI-based present work *L. haematopterus* (LC) was found to be a well-supported *Lethrinus* clade aside from the two trophic lineages and then ancestor to these lineages. The author of the current work phylogenetically (PM and NJ methods, 500 and 1000 replications, respectively) analyzed 34 cytochrome b sequences (1140 bp) of 20 *Lethrinus* species (Table 9) retrieved from NCBI database[59,60]. It is postulated that *L. miniatus* did not position as ancestor of that genus, only separated with *L. erythropterus* in one cluster in one of *Lethrinus* lineages (Fig. 8).

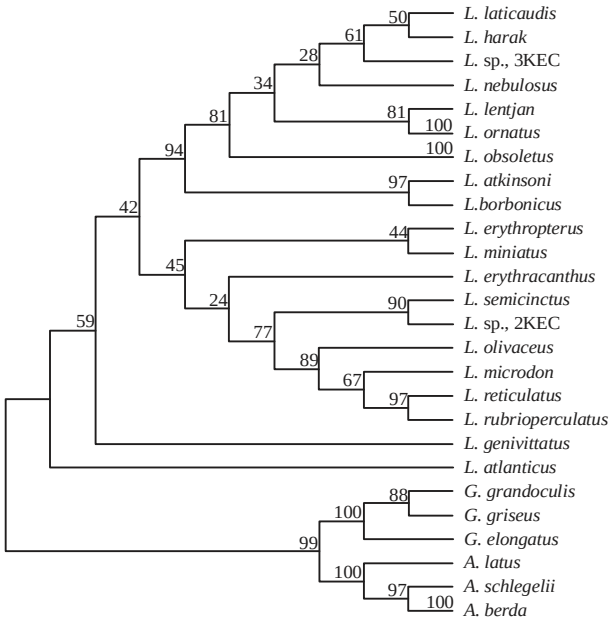

Fig. 8: Maximum parsimony-based phylogenetic tree of 24 cytochrome b sequences (1140 bp) of 20 *Lethrinus* species with 10 other sequences (1140 bp) of 6 species of genera: *Gymnocranius* and *Acanthopagrus* as outgroup (1137 nucleotide positions are included in final data set, 500 replications, booted). Sequences retrieved from NCBI databases

Table 9: List of cytochrome b sequences of *Lethrinus* species and outgroups used in their phylogenetic tree retrieved from GenBank database

1	gi|21388806|gb|AF381263.1| *Lethrinus rubrioperculatus*
2	gi|21388818|gb|AF381269.1| *Lethrinus nebulosus*
3	gi|21388812|gb|AF381266.1| *Lethrinus miniatus*
4	gi|21388796|gb|AF381258.1| *Lethrinus harak*
5	gi|21388816|gb|AF381268.1| *Lethrinus borbonicus*
6	gi|21388814|gb|AF381267.1| *Lethrinus lentjan*
7	gi|21388790|gb|AF381255.1| *Lethrinus atkinsoni*
8	gi|21388810|gb|AF381265.1| *Lethrinus microdon*
9	gi|21388786|gb|AF381253.1| *Lethrinus reticulatus*
10	gi|300116377|emb|AM944836.2| *Acanthopagrus berda*
11	gi|300116381|emb|AM944838.2| *Acanthopagrus berda*
12	gi|300116375|emb|AM944835.2| *Acanthopagrus berda*
13	gi|300116379|emb|AM944837.2| *Acanthopagrus berda*
14	gi|300116383|emb|AM944839.2| *Acanthopagrus berda*
15	gi|219937694|emb|AM265581.1| *Acanthopagrus latus*
16	gi|67772403|gb|DQ069319.1| *Acanthopagrus schlegelii*
17	gi|21388804|gb|AF381262.1| *Lethrinus* sp., 2-KEC-2001
18	gi|21388798|gb|AF381259.1| *Gymnocranius griseus*
19	gi|21388784|gb|AF381252.1| *Lethrinus olivaceus*
20	gi|21388792|gb|AF381256.1| *Lethrinus* sp., 3-KEC-2001
21	gi|21388782|gb|AF381251.1| *Lethrinus laticaudis*
22	gi|21388832|gb|AF381276.1| *Lethrinus semicinctus*
23	gi|21388808|gb|AF381264.1| *Lethrinus atlanticus*
24	gi|21388800|gb|AF381260.1| *Gymnocranius elongatus*
25	gi|21388830|gb|AF381275.1| *Gymnocranius grandoculis*
26	gi|21388794|gb|AF381257.1| *Lethrinus erythracanthus*
27	gi|748584748|dbj|AB355916.1| *Lethrinus obsoletus*
28	gi|21388788|gb|AF381254.1| *Lethrinus ornatus*
29	gi|13183284|gb|AF240751.1| *Lethrinus ornatus*
30	gi|157736061|ref|NC 009855.1| *Lethrinus obsoletus*
31	gi|21388824|gb|AF381272.1| *Lethrinus genivittatus*
32	gi|21388828|gb|AF381274.1| *Lethrinus erythropterus*
33	gi|139000570|dbj|AP009165.1| *Lethrinus obsoletus*
34	gi|21388822|gb|AF381271.1| *Lethrinus obsoletus*

The differences between the four categories: *L. haematopterus*, the other two lineages and the out groups (*Gymnocranius*) were emphasized to be significant by AMOVA analyses of their haplotypes. The phylogenetic analyses of the corresponding 48 haplotypes reflect also the same phylogenetic-based pattern of relationship between these lineages and their ancestor. According to the results based on cytochrome b and COI, which species of *L. haematopterus* and *L. maniatus*, one can postulated as ancestor to *Lethrinus* species. In the present study, *L. maniatus* was only clustered with *L. olivaceus* (LC) and L. sp. (LC) in all analyses using different methods. So, *L. haematopterus* will be considered as the ancestor of *Lethrinus* species especially the DNA barcode, COI is recommended as a well-established method by many researchers in the evolutionary and phylogenetic studies as well as in global market for fisheries and aquaculture products[24,25,28,51,53,61,62].

The *Lethrinus* genus, *Gymnocranius* with HC-trophic type positions in all analysis as a sister group of genus *Lethrinus* in the present study. Generally one can emphasized on hypothesis of Lo Galbo *et al.*[2] that the two primary trophic types (HM and LC) are evolved from a high-bodied conical-toothed (HC) ancestor. The placement of *L. miniatus* in lineage 2 (CL-lineage) of the present study may be explained as a support of this hypothesis. In the study of Lo Galbo *et al.*[2], *L. erythropterus* (HM) and *L. erythracanthus* (HC) were grouped within LC-lineage whereas *L. genivittatus* (LC) and *L. atlanticus* (HC) were clustered within HM-lineage. These four *Lethrinus* species are not represented in the current COI-database. It is expected that such overlapping of these species could not be recorded if they are represented in COI-database. These findings of *L. haematopterus* (only restricted to temperate waters in East Asia) and *L. miniatus* with antitropical distribution[1] referred to a temperate water form to be the ancestor of the genus *Lethrinus*[2]. Finally, on the basis of aforementioned discussion, do genetic analysis of different mitochondrial genes and/or of different genes of the whole genome lead to different gene-based ancestors or one one genome-based ancestor to a given genus Like *Lethrinus*? A question needs to be answered.

CONCLUSION

According to the current study, one can concluded the following points: (1) DNA sequences databases and sources must be validated and filtered to prevent paraphyla in phylogenetic studies and to correct misidentification of specimens, (2) During construction of phylogenetic trees, different statistical methods should be considered, (3) Based on COI sequences released from BOLD system database, genus *Lethrinus* exhibited two lineages with *L. haematopterus* as ancestor, (4) This taxonomic status of *Lethrinus* was in accordance to the three trophic types (LC, HM and HC) and (5) COI-based analyses is moe better than those based on cytochrome b and mini-DNA barcoding.

SIGNIFICANT STATEMENT

The current study is important due to:

- The taxonomic morphometric-related problems in *Lethrinus*
- The appearing of DNA barcoding as a valid new technology in solving phylogenetic problems
- The application of this technology on *Lethrinus* species for determination of evolutionary trends in corresponding of trophic evolution

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