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Phylogeny of Ponyfishes from Coastal Waters of the South China Sea

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Abstract

Leiognathid fishes were collected during several series of trawl surveys carried out in the coastal waters of the South China Sea on the east coast of Peninsula Malaysia. Taxonomic revision for leiognathid fishes is probably needed because diagnostic characters have been described without a broad comparison among existing species, resulting in nomenclatural problems. The phylogenetic relationship among 14 morphospecies of leiognathid fishes were inferred from 16S mitochondrial rRNA gene sequences. The results showed that molecular phylogenetic positions of the fishes agreed well with morphological delineation. The only exception was *Leiognathus* sp. "Philippines" AY541655 that was more closely affiliated to *Nuchequula* spp. than to other *Leiognathus* spp. Neighbor-joining analysis suggested that the genus *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic. *Leiognathus equulus* and *Leiognathus longispinis* formed the base of the other clades. *Leiognathus daura* formed a sister group to *Nuchequula* species. *Leiognathus splendens* and *Leiognathus jonesi* formed sister taxa to *Photopectoralis*. *Gazza* was a sister group to *Secutor* but with low confidence value. *Photoplagios* and *Secutor* both formed two distinct subclades. It is suggested that analysis of 16S mitochondrial rDNA would be highly valuable to resolve some of the outstanding taxonomic problems in the family Leiognathidae.

Key words: Leiognathidae · Phylogenetic analysis, 16S mitochondrial rRNA gene, Fish phylogenetics, Malaysia

INTRODUCTION

Leiognathid fishes (family Leiognathidae), commonly known as slipmouths or ponyfishes, are widely distributed in the coastal waters of sub-tropical and tropical regions [7]. In Malaysia, it is known as "kekek", a moniker based on the chirping sound the fish makes. These fishes commonly inhabit turbid coastal waters of poor visibility such as shallow coastal waters, estuaries and mangrove areas [28, 32], although they occasionally venture into freshwater reaches of rivers. They are demersal fishes that usually form large mixed feeding schools of a few to several species on the shallow water sea floor. Ponyfishes are commercially important 'by-catch' fishes in Malaysian fisheries. In some parts of the country ponyfishes along with other fishes such as Mullidae and Gerreidae are processed into a popular snack locally known as 'fish satay' [19].

The leiognathids are characterized by having a highly protractible mouth and a circumesophageal light organ in which are harbored the luminescent bacterium *Photobacterium leiognathi* as the predominant symbiont and *Photobacterium mandapamensis* as cosymbiont [8]. At present, the family comprises six genera namely *Gazza*, *Leiognathus*, *Secutor*, *Photopectoralis*, *Photoplagios* and *Nuchequula* [3]. There are 18 species of *Leiognathus*, five species of *Gazza*, six species of *Secutor*, four species of *Photopectoralis*, eight species of *Photoplagios* and five species of *Nuchequula* [1, 3, 26, 28]. *Gazza*, *Leiognathus* and *Secutor* are differentiated by the direction of mouthpart protraction and teeth form. The mouth of *Gazza* protracts forward and there are distinct caniniform teeth on both jaws. The mouth of *Leiognathus* protracts forward and downward and there are small weak teeth on both jaws. *Secutor* has an upwardly protractile mouth with small weak teeth on both jaws [18]. The morphology of the protractile mouth type has been suggested as phylogenetically informative [6].

The sexually dimorphic luminescent system is a unique character in leiognathids and has been widely studied with regard to its evolution and diversification [28]. The sexually dimorphic light organs were proposed as phylogenetically informative. Based on such studies two new genera, namely *Photopectoralis* and *Photoplagios*, were recently described [28]. Males of all species within these two genera are easily distinguished from each other by the size, shape, and placement of the transparent lateral flank patch, pectoral-axil patch or external mid-lateral stripe [24, 26, 28]. However, these features are not present in females. *Nuchequula* is characterized by the presence of a distinct saddle-shaped nuchal marking and by the presence of a pigment-free, mitten-shaped region posteroventral to the pectoral-fin base [1].

Leiognathidae is in need of taxonomic revision because diagnostic characters have been poorly defined, or not defined at all, or described without a broad comparison to existing species when naming a new species. The type specimen for many species have never been deposited, are now lost or in poor condition, or alleged extant types do not match the corresponding original descriptions [25]. Leiognathids are difficult to diagnose and identify because they are morphologically conservative fishes across genera and may form species complex [28]. For these reasons Leiognathidae taxonomy is plagued by numerous

nomenclatural problems, which not only contribute to frequent misidentifications but also to the creation of ''wastebasket'' species [1, 25]. Several analyses of these fishes have been carried out focusing on morphology [9, 10, 12, 24, 26, 33]. Family-level phylogenetic analyses have provided a better understanding of the relationship of leiognathids at the generic and species levels [1, 6, 24, 27, 28]. However, the monophyly of the six genera has yet to be evaluated, owing to a lack of information on the phylogenetic relationships among the genera and species. The aim of the current study was to analyze the molecular phylogeny and the congruence of molecular data with morphological descriptions of leiognathid fishes present in Malaysia waters.

MATERIALS and METHODS

Fourteen morphospecies of leiognathid fishes were included in this study (Fig. 1). Samples were collected from the waters of Perhentian Island and Tinggi Island in the South China Sea (Table 1). Three individuals of each morphospecies were used in this study. Morphospecies identification was based on James [7], Masuda *et al.* [16], Mohsin and Ambak [20], Mansor *et al.* [15], Matsuura *et al.* [18], Woodland *et al.* [32], Yamashita and Kimura [33], Nakabo [21], Kimura and Matsuura [11], Kimura *et al.* [10], Matsuura and Kimura [17], Sparks [24], Chakrabarty and Sparks [1] and Sparks and Chakrabarty [26].

Fish tissues were preserved in absolute ethanol prior to extraction of DNA. Total genomic DNA was extracted from dorsolateral muscle using a modified CTAB method [5]. PCR was used to amplify a segment (~600 bp) of the 16S mitochondrial ribosomal RNA gene. DNA amplifications were performed in 50µL volumes containing 5µL of 10X PCR buffer, 3µL of 25 mM MgCl2, 1µL of 10 mM dNTPs (Promega, USA), 2.5µL of 10pmol/µl of each primer, 5µL of template genomic DNA, 2µL of 2µ/µL Taq polymerase (Promega, USA) and 29µL of ddH_2O. To amplify and sequence the 16S mitochondrial rDNA fragment, the primers 16S ar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16S br-H (5'-CCGGTCTGAACTCAGA TCACGT- 3') [14, 22] were used.

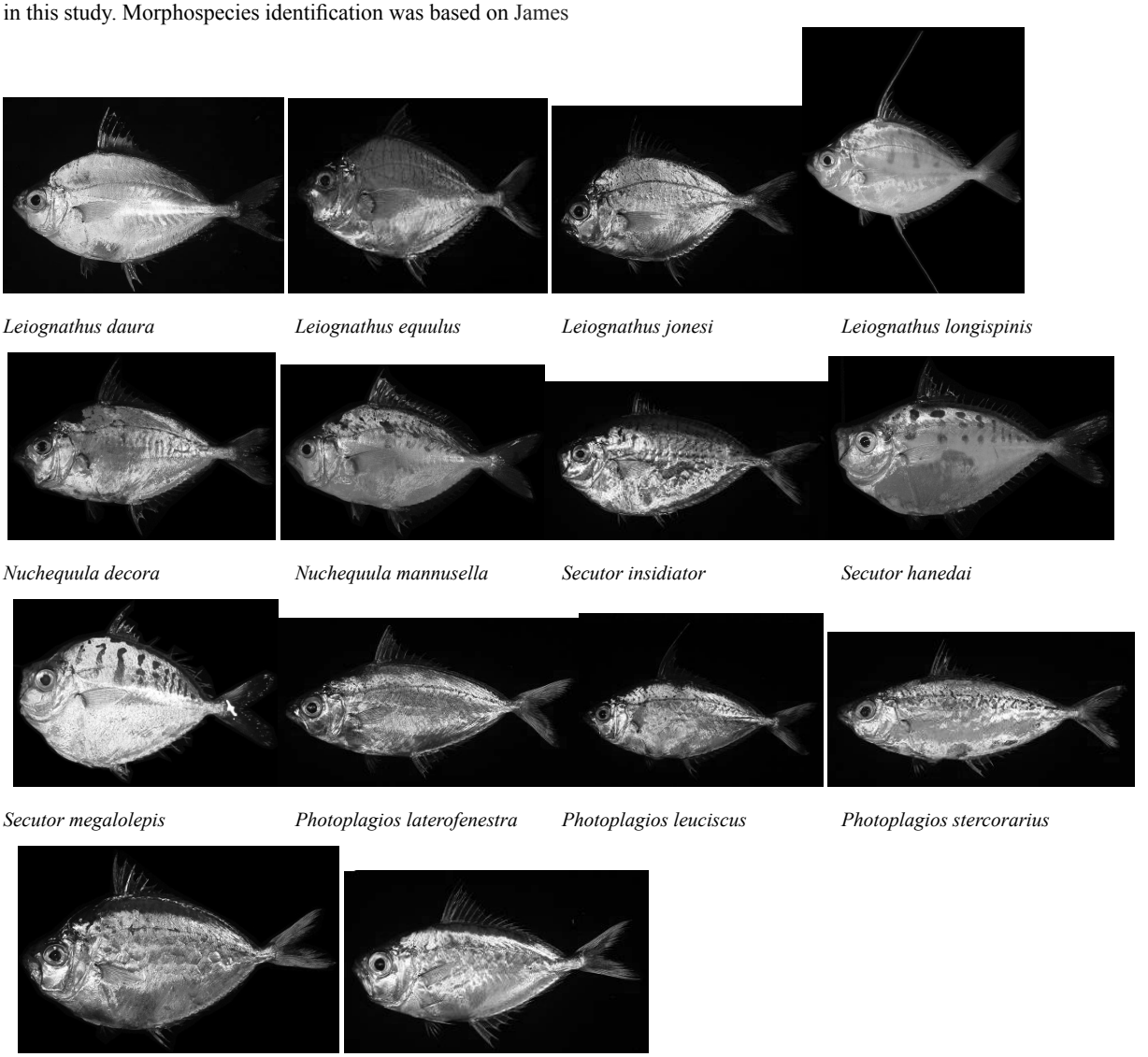

Figure 1 The fourteen species of leiognathids selected for molecular phylogenetic analysis in the study

Table 1 Locations and dates of sampling for ponyfishes used in this study

Location	Collection time
Perhentian Island	
N05° 59.863: E102° 46.623' - N06° 04.467': E102° 38.895'	02.09.2006
N06° 00.950: E102° 38.040' - N06° 02.227': E102° 42.119'	03.09.2006
N05° 50.440: E102° 47.240' - N05° 49.963': E102° 48.488'	04.09.2006
Tinggi Island	
N02° 13.830: E103° 59.280' - N02° 12.530': E103° 59.057'	17.07.2007
N02° 15.452: E103° 59.829' - N02° 13.314': E104° 00.099'	18.07.2007
N02° 11.444: E104° 00.441' - N02° 10.945': E104° 02.121'	19.07.2007

Amplification was carried out over 30 cycles in a PTC-150 MiniCycler™ (MJ Research Inc, USA). The thermal cycle profile was as follows: 6 min at 96°C for initial denaturation, 45 sec at 95°C for denaturation, 1 min 30 sec at 47 °C for annealing, 1 min 30 sec at 72°C for extension and 7 min at 72°C for additional terminal extension. The PCR product was purified using QIAquick purification kit (Qiagen Inc, USA) according to the manufacturer's recommended protocol. Purified PCR product was directly cycle-sequenced using the original amplification primers and the ABI PRISM BigDye® Terminator v3.0 Cycle Sequencing kit. Sequencing was performed on an ABI 377 automated sequencer (PE Applied Biosystem Inc, USA).

Sources of sequence data for phylogenetic analysis are shown in Table 2. Multiple sequence alignment for forward reactions was carried out using CLUSTALX version 1.81 [31], and subsequently aligned by eye. Modeltest 3.7 [23] was used to estimate the base frequencies, nucleotide substitution rate, proportion of invariable sites and gamma distribution shape parameter. Phylogenetic relationships were analyzed by neighbour-joining (NJ) and maximum parsimony (MP) methods using PAUP* version 4.0b10 [29]. Heuristic search NJ was performed using random sequence additions (n=10) and tree bisection-reconnection (TBR) branch swapping. Bootstrap support values for individual nodes were obtained from 1000 replicates. Heuristic search MP was performed with 1000 replications and 10 random stepwise additions of taxa. Consistency indices (CI), retention indices (RI), rescaled consistency indices (RC) and homoplasy (HI) [4, 13] were computed in PAUP* version 4.0b10.

RESULTS and DISCUSSION

A single consensus tree was generated from the analysis of the 16S mitochondrial rRNA gene sequences (Fig. 2). Results of the Modeltest analyses showed that the substitution model of TrN+I+G [30] provided the best fit to the data, selected by hierarchical likelihood ratio test (hLRT). Model parameters estimated were as follows: empirical base frequencies A = 0.3498, C = 0.2653, G = 0.1675 and T = 0.2174; nucleotide substitution rate [A-C] = 1.0000, [A-G] = 6.2397, [A-T] = 1.0000, [C-G] = 1.0000, [C-T] = 9.2525 and [G-T] = 1.0000; proportion of invariable sites (I) = 0.4102; gamma distribution shape parameter (α) = 0.5200. All 472 nucleotide characters (295 constant; 29 parsimony-uninformative; 148 parsimony-informative) from 50 ingroup and 3 outgroup taxa were analyzed simultaneously. This resulted in one most-parsimonious tree with a length of 472 steps (CI = 0.5636; RI = 0.8577; RC = 0.4834; HI = 0.4364).

Gerreids and carangoids, currently hypothesized to be close relatives of ponyfishes [28] were used as outgroups. In NJ reconstruction, the monophyly of Leiognathidae was strongly supported by a bootstrap value of 100%. Within Leiognathidae six clades were recovered. *Nuchequula*, *Photoplagios*, *Photopectoralis*, *Gazza* and *Secutor* each formed a clade with bootstrap support of 94%, <50%, 83%, 100% and 81% respectively. *L. equulus* and *L. longispinis* formed the base of the other clades supported by a moderate bootstrap value of 55%. *Leiognathus daura* formed a sister group to *Nuchequula* species, with 67% bootstrap support. *Photoplagios* formed two distinct subclades, one comprising *P. stercorarius* (bootstrap value 99%) and another comprising *P. rivulatus*, *P. laterofenestra* and *P. leuciscus* (bootstrap value 88%). However, bootstrap support for the relationship within these two subclades was weak. The NJ analysis placed *Leiognathus splendens* and *Leiognathus jonesi* as sister taxa to *Photopectoralis* (95% bootstrap value). *Gazza* formed a sister group to *Secutor* but with low statistical support. *Secutor* formed two distinct subclades, one comprising *Secutor indicus* and *Secutor insidiator* (bootstrap value 100%) and another comprising *Secutor hanedai* and *Secutor megalolepis* (bootstrap value 90%). The NJ tree suggested that the genus *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic. Similar results were obtained by Ikejima *et al.* [6], Sparks and Dunlap [27] and Sparks *et al.* [28] using a combination of different genetic markers. Our study showed that analysis of just the 16S mitochondrial rDNA could be sufficient to determine the Leiognathidae phylogenetic relationship.

In general the molecular phylogenetic positions of the fishes studied agreed well with morphological delineation. The only exception was *Leiognathus* sp. "Philippines" (Genbank accession code AY541655) that was more closely affiliated to *Nuchequula* spp. than to other *Leiognathus* spp. This species was examined by Sparks *et al.* [28] and was determined to represent an undescribed species. In our opinion, this undescribed species was most probably a species of *Nuchequula* because there was only one base difference in the 16S mt-rDNA sequence with our specimen of *Nuchequula mannusella*. This speculation was also supported by the position of the Philippines specimen on the phylogenetic tree. Currently corresponding nucleotide sequences for two *Nuchequula* species, namely *Nuchequula blochii* and *Nuchequula pan* are not yet available. It is probable that the Philippines specimen is any one of these species.

Sparks *et al.* [28] suggested that *Leiognathus fasciatus* and *Leiognathus equulus* each formed independent species complex. Both do not appear to be internally or externally sexually dimorphic with regard to the light organ system. Phylogenetic analysis in this study indicated that *Leiognathus longispinis*, which also do not exhibit sexual dimorphism of the light organ, formed a sister group to *Leiognathus equulus*. This result supports the suggestion that the light organ morphism is phylogenetically informative.

Table 2 Sources of sequence data for phylogenetic analysis

Species	Sequence accession number	Reference
Carangoides equula	AY541670	Sparks and Dunlap (2004)
Gerres abbreviatus	AY541667	Sparks and Dunlap (2004)
Gerres equulus	AY541668	Sparks and Dunlap (2004)
Leiognathus equulus "Philippines"	AY541653	Sparks and Dunlap (2004)
Leiognathus equulus "Singapore"	AY541654	Sparks and Dunlap (2004)
Leiognathus equulus "Japan"	DQ027947	Sparks et al. (2005)
Leiognathus equulus "Taiwan"	DQ027948	Sparks et al. (2005)
Leiognathus daura "Sri Lanka"	DQ027954	Sparks et al. (2005)
Leiognathus daura "Sri Lanka"	DQ027955	Sparks et al. (2005)
Leiognathus sp. "Philippines"	AY541655	Sparks and Dunlap (2004)
Nuchequula decora "Australia"	DQ027956	Sparks et al. (2005)
Nuchequula decora "Sri Lanka"	DQ027957	Sparks et al. (2005)
Photoplagios stercorarius "Philippines"	AY541663	Sparks and Dunlap (2004)
Photoplagios rivulatus "Japan"	AY541661	Sparks and Dunlap (2004)
Photoplagios leuciscus "Philippines"	AY541657	Sparks and Dunlap (2004)
Photoplagios leuciscus "Madagascar"	DQ027964	Sparks et al. (2005)
Photoplagios leuciscus "Japan"	DQ027965	Sparks et al. (2005)
Leiognathus splendens "Philippines"	AY541662	Sparks and Dunlap (2004)
Leiognathus jonesi "Philippines"	AY541656	Sparks and Dunlap (2004)
Photopectoralis bindus "Philippines"	AY541651	Sparks and Dunlap (2004)
Photopectoralis sp. "Japan"	DQ027961	Sparks et al. (2005)
Photopectoralis sp. "Taiwan"	DQ027962	Sparks et al. (2005)
Gazza minuta "Philippines"	AY541649	Sparks and Dunlap (2004)
Gazza minuta "Sri Lanka"	DQ027936	Sparks et al. (2005)
Gazza minuta "Sri Lanka"	DQ027937	Sparks et al. (2005)
Gazza minuta "Philippines"	DQ648428	Dunlap et al. (2007)
Secutor indicius "Philippines"	AY541665	Sparks and Dunlap (2004)
Secutor indicius "Sri Lanka"	DQ027970	Sparks et al. (2005)
Secutor cf. *insidiator* "Sri Lanka"	DQ027971	Sparks et al. (2005)
Secutor megalolepis "Philippines"	AY541666	Sparks and Dunlap (2004)
Secutor megalolepis "Australia"	DQ027972	Sparks et al. (2005)
Secutor megalolepis "Philippines"	DQ648432	Dunlap et al. (2007)
Leiognathus daura "Tinggi Island"	EU366333	This study
Leiognathus jonesi "Tinggi Island"	EU366335	This study
Leiognathus longispinis "Tinggi Island"	EU366331	This study
Nuchequula decora "Tinggi Island"	EU366343	This study
Nuchequula mannusella "Tinggi Island"	EU366330	This study
Photopectoralis bindus "Tinggi Island"	EU366344	This study
Gazza minuta "Tinggi Island"	EU366347	This study
Secutor hanedai "Tinggi Island"	EU366332	This study
Secutor megalolepis "Tinggi Island"	EU366337	This study
Photoplagios leuciscus "Tinggi Island"	EU366348	This study
Photoplagios leuciscus "Tinggi Island"	EU366340	This study
Photoplagios stecorarius "Tinggi Island"	EU366345	This study
Leiognathus equulus "Perhentian Island"	EU366341	This study
Secutor insidiator "Perhentian Island"	EU366336	This study
Photoplagios laterofenestra "Perhentian Island"	EU366342	This study
Nuchequula decora "Perhentian Island"	EU366328	This study
Photopectoralis bindus "Perhentian Island"	EU366329	This study
Photoplagios stecorarius "Perhentian Island"	EU366334	This study
Leiognathus jonesi "Perhentian Island"	EU366346	This study
Gazza minuta "Perhentian Island"	EU366338	This study
Photoplagios leuciscus "Perhentian Island"	EU366339	This study

Photoplagios formed two distinct subclades, in agreement with the features of the light organ system. *P. rivulatus*, *P. laterofenestra* and *P. leuciscus* have an expansive transparent flank patch and the dorsolateral lobes of the light organ are hypertrophied and extend posteriorly into the air bladder. In contrast, *P. stercorarius* has a transparent mid-lateral stripe and the lobes of the light organ do not extend into the air bladder. Both subclades have clear lateral lining of the air bladder [24, 27, 28]. *Secutor* also formed two subclades, also in agreement with morphology. *S. indicius* and *S. insidiator* have body sizes almost one time bigger than *S. hanedai* and *S. megalolepis* [32]. *Secutor interruptus* and *Secutor ruconius* have small sized body and are probably sister taxa to *S. hanedai* and *S. megalolepis*. Sparks *et al.* [28] showed that *Secutor* cf. *ruconius* was a sister taxa to *S. hanedai* and *S. megalolepis*. These results suggested that body size is an important characteristic for preliminary identification of *Secutor* species.

Another interesting species was *Photoplagios leuciscus*. Specimens caught in the waters of Tinggi Island can be divided into two morphotypes (Fig. 3a,b). One group displayed faint speckles and had 100% sequence similarity with Philippines specimen AY541657 and differed by seven bases with

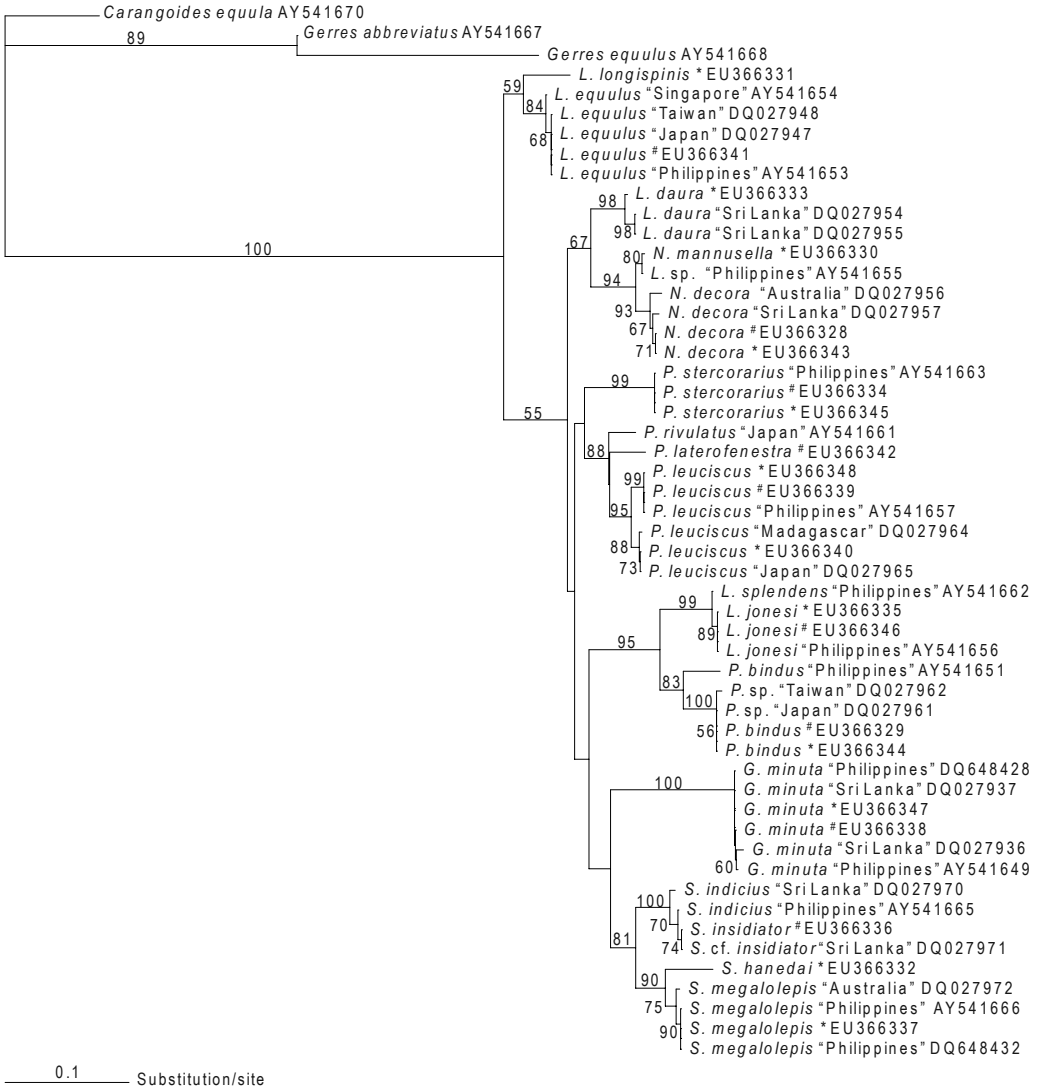

Figure 2 Neighbour-joining tree of leiognathids based on 16S mt-rDNA sequences. The tree included sequence data of Leiognathidae available from GenBank. Numbers at nodes represent bootstrap support. * represents samples collected from Tinggi Island and # were samples from Perhentian Island. *Carangoides equula*, *Gerres equulus* and *Gerres abbreviatus* were used as outgroups.

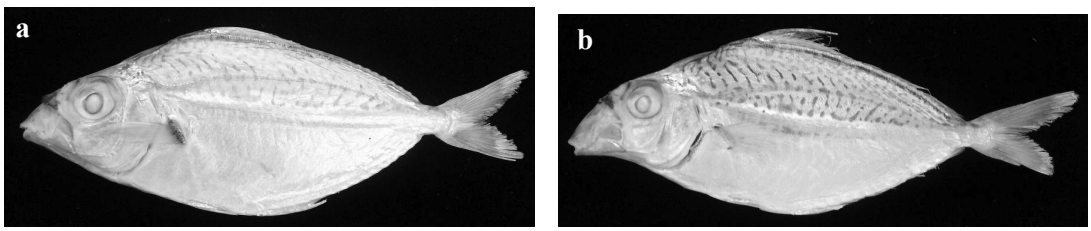

Figure 3 Variation in stripe patterns of *Photoplagios leuciscus* collected from Tinggi Island. **a.** Body displays faint speckles with fine lines and small spots. **b.** Body displays distinct speckles with fine lines and small spots.

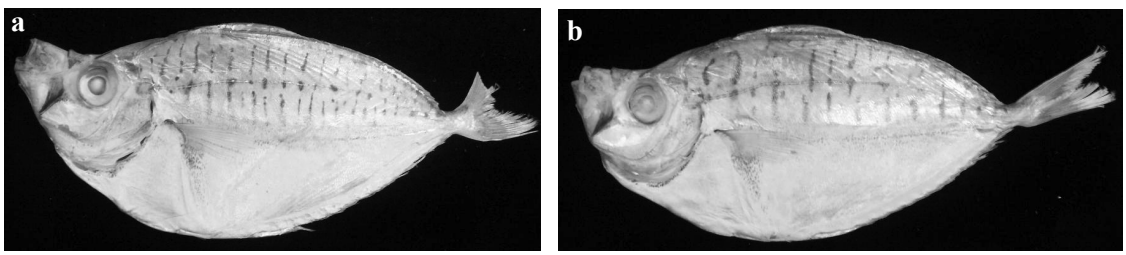

Figure 4 Variation in stripe patterns of *Secutor insidiator* collected from Perhentian Island. **a.** A series of 20 dark vertical bars terminate above the lateral line and resume below it, with rows of dots along lateral line corresponding to the cross bars. **b.** A series of 12 dark vertical bars and spots extending to a little below the lateral line.

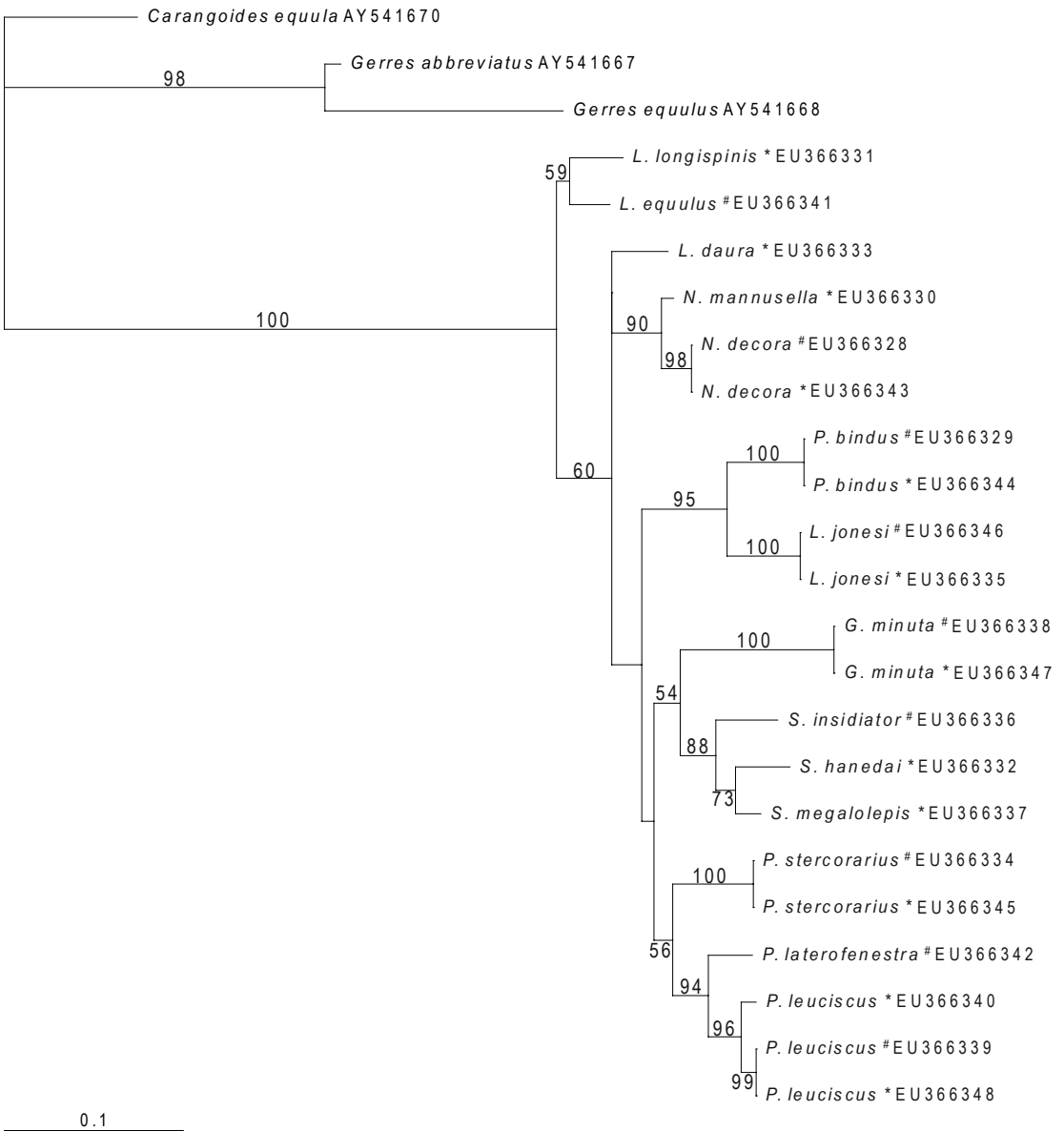

Figure 5 Neighbour-joining tree of leiognathids based only on 16S mt-rDNA sequences obtained in this study. Numbers at nodes represent bootstrap support. ***** represents samples collected from Tinggi Island and # were samples from Perhentian Island. *Carangoides equula*, *Gerres equulus* and *Gerres abbreviatus* were used as outgroups.

Madagascar specimen DQ027964. The second group displayed distinct speckles and had 100% sequence similarity with Japan specimen DQ027962 and one base difference with Madagascar specimen DQ027964. High sequence similarity between these *P. leuciscus* specimens from widely separated geographical areas suggested that the species has wide distribution and migration to enhance gene exchange. Alternatively the results suggested that the mt-rDNA in this species evolves very slowly.

Malaysian specimens of *Photopectoralis bindus* shared 100% sequence similarity to unidentified *Photopectoralis* species from Okinawa and 99% sequence similarity to unidentified *Photopectoralis* species from Taiwan. However, there were 22 base differences with *P. bindus* "Philippines" AY541651 even though morphologically they were identical. Data available to date showed no other Leiognathidae species groups together with the *P. bindus* clade. Thus the specimens from Okinawa and Taiwan were most probably *P. bindus.*

Malaysian specimens of *Secutor insidiator* (Fig. 4a,b) that showed similar dorsolateral markings to *S. indicius* and *S. insidiator* [32], all shared 100% sequence similarity to *Secutor* cf. *insidiator* "Sri Lanka" but had one base difference with *S. indicius* "Philippines" and six bases difference with *S. indicius* from Sri Lanka. Thus in this case two different morphospecies had identical or almost identical sequence. However, since morphological characteristic has higher priority than molecular data in fish taxonomy and since *S. insidiator* was named earlier than *S. indicius*, so the name *S. insidiator* was used in the current study. We are also of the opinion that these two species should be combined, although more specimens need to be examined to support this.

A phylogenetic tree using only sequences of 16S mitochondrial rRNA gene obtained in this study was generated (Fig. 5) to describe our own samples for comparison with published reports. The results showed that there were minor

differences from the tree obtained using all available sequence data (Fig. 2). The clade containing *Photoplagios* was more closely related to *Gazza* and *Secutor* in the subset tree. Also, *P. bindus* and *L. jonesi* formed the base of the *Gazza*, *Secutor* and *Photoplagios* clades. *L. daura* was no longer a sister taxa to *Nuchequula* species and also formed a trifurcation with 60% bootstrap support. *L. daura* and *Nuchequula* have a similar pattern of black blotch at the distal half of the fin membrane between the second and sixth dorsal fin spines. However, *L. daura* is different from *Nuchequula* by the presence of a distinct saddle-shaped nuchal marking in the latter. *Nuchequula* also posseses a pigment-free mitten-shaped region posteroventral to the pectoral fin base that is absent in *L. daura*. As shown in Fig. 5 there was stronger bootstrap support (>50%) for the relationship of *Gazza* with *Secutor*, *Photoplagios stercorarius* with *Photoplagios* group and *P. laterofenestra* with *P. leuciscus*. *L. equulus* and *L. longispinis* formed the base of the other clades. However, the phyly of genera was the same from the analysis of both datasets. *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic.

In conclusion, this study has shown that mitochondrial 16S rDNA sequence is a very good marker for phylogenetic analysis of the Leiognathidae. The molecular phylogenetic positions of the fishes were in congruence with morphological delineation. *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic. These results, and those from previous studies, suggested that a more robust morphological criterion, coupled with relevant molecular data should be employed to resolve taxonomic uncertainties among this group of fishes.

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