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MOLECULAR PHYLOGENY OF LEIOGNATHIDAE IN THE WATERS OF PERHENTIAN ISLANDS, TERENGGANU, MALAYSIA

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ABSTRACT

Several series of trawl surveys were carried out in the waters of Perhentian Islands using commercial trawlers. 16S mitochondrial rRNA gene sequences were used to infer the phylogenetic relationships among nine morphospecies of leiognathids. The results showed that the genus *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic. The molecular phylogenetic positions of the leiognathids studied were identical with morphological delineation, except for *Photoplagios stercorarius*. Branch of *P. stercorarius* was placed between *Photoplagios* spp. clade and clade of *Secutor* and *Gazza*. *P. stercorarius* was more affiliated to genus *Photoplagios* morphologically however have slight different features of light organ system compared with others three *Photoplagios* sp. caught in this study. It is probable that two distinct subclades occur in genus *Photoplagios*. *Leiognathus equulus* formed the base of the other leiognathids. *Leiognathus splendens* and *Leiognathus jonesi* formed a sister taxa to *Photopectoralis* species. *Gazza* formed a sister taxa to *Secutor* and *Nuchequula* formed a sister taxa to the group of trifurcation but both with low bootstrap support. This study has shown that 16S mitochondrial rDNA is a good marker for phylogenetic analysis of the Leiognathidae.

Keywords: Leiognathidae, Molecular phylogeny; 16S mitochondrial rRNA gene

INTRODUCTION

Leiognathids (family Leiognathidae), is most widely known as slimys, slipmouths or ponyfishes (Nelson, 1994). Chiefly, the common name of this fish in Malaysia is "kekek", a moniker based on the chirping sound the fish makes. They are demersal fishes that widely distributed in the coastal waters of sub-tropical and tropical regions (James, 1984), ordinarily inhabit turbid coastal waters of poor visibility such as mangrove areas, estuaries and shallow coastal waters although once in a while they venture into freshwater reaches of rivers (Sparks *et al.*, 2005; Woodland *et al.*, 2001).

These fishes normally form heavy 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 certain parts of the country, ponyfishes along with other fishes such as Gerreidae and Mullidae are processed into a popular snack locally known as 'fish satay' (Mazlan and Seah, 2006).

This fish species is generally recognized by its protractible mouth either in an upward, forward or downward direction. There are approximately 46 species containing in six genera namely *Gazza*, *Leiognathus*, *Secutor*, *Photopectoralis*, *Photoplagios* and *Nuchequula* (Eschmeyer, 2007). The direction of mouthpart protraction and teeth form are used to differentiate *Gazza*, *Leiognathus* and *Secutor* (Matsuura *et al.*, 2000) and has been suggested as phylogenetically informative (Ikejima *et al.*, 2004). The sexually dimorphic luminescent system is a unique character in leiognathids and has been widely studied with regard to its evolution and diversification (Sparks *et al.*, 2005). The sexually dimorphic light organs were proposed as phylogenetically informative. Based on such studies two new genera, namely

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Photopectoralis and *Photoplagios*, were recently described (Sparks *et al.*, 2005). *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.

Leiognathids are in need of taxonomic revision because these fishes are morphologically conservative fishes across genera and may form a species complex. And yet, the poor defined of species, poor condition of specimen and described new species without broad comparison created frequent misidentifications and form a great taxonomically problematic now. Several analyses of these fishes have been carried out focusing on morphology (Kimura *et al.*, 2000; Yamashita and Kimura, 2001; Kimura *et al.*, 2003; Kimura *et al.*, 2005) and molecular phylogeny (Ikejima *et al.*, 2004; Sparks and Dunlap, 2004; Sparks *et al.*, 2005; Sparks, 2006). The aim of this study is to analyze the phylogenetic relationships and the identical between molecular data and morphological descriptions of leiognathids present in Perhentian Island waters.

MATERIALS AND METHODS

Leiognathids were collected during several series of trawl surveys carried out in the coastal waters of Perhentian Islands using commercial trawlers within N06° 04.467: E102° 38.895' - N06° 00.950': E102° 38.040', N05° 50.440': E102° 47.240' – N05° 49.963': E102° 48.488'. A total of 10 hauls were made throughout the study, each trawling lasted about 3 hours at a towing speed at 2.0 – 3.0 knot. Otter trawl net was deployed throughout the study to catch the leiognathids at depths ranging from 50 to 70 feet. The trawl was equipped with a 1¼ inches cod end mesh size. All catches were sorted in accordance to the standard protocol listed by Sparre and Venema. Fresh sub-samples were kept in the ice prior to further biological investigation at field-laboratory station. Fishes tissues were dissected and preserved in absolute ethanol prior to DNA extraction. Tagged specimens of fishes were photographed for their whole body. All sub-samples collected were then fixed in 10% formalin during field study and later transferred into 70% alcohol prior to the laboratory.

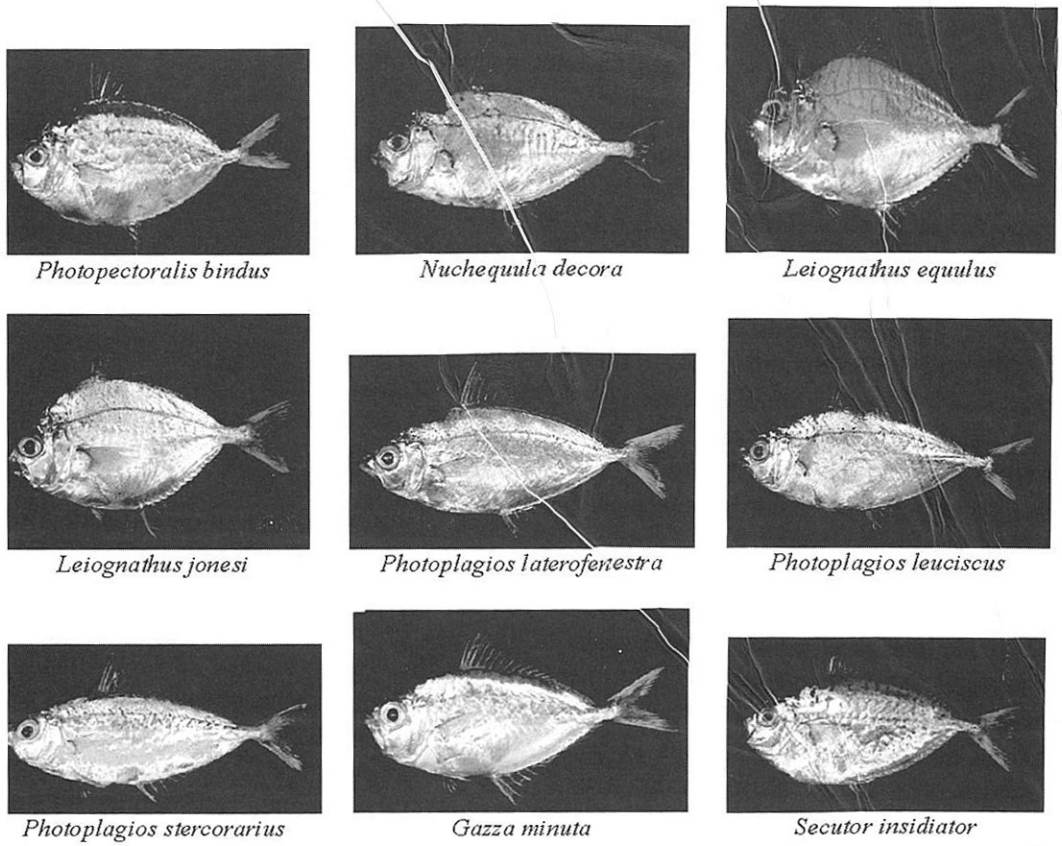

Figure 1.The nine species of leiognathids selected for molecular phylogenetic analysis in the study.

Nine morphospecies of leiognathids were included in this study (Fig. 1). Morphospecies identification was based on James (1984), Masuda *et al.* (1984), Mohsin and Ambak (1996), Mansor *et al.* (1998), Matsuura *et al.* (2000), Woodland *et al.* (2001), Yamashita and Kimura (2001), Nakabo (2002), Kimura and Matsuura (2003), Kimura *et al.* (2005), Matsuura and Kimura (2005), Sparks (2006), and Sparks and Chakrabarty (2007).

Three individuals of each morphospecies were used to analyze the phylogenetic relationships. Total genomic DNA was extracted from dorsolateral muscle using a modified CTAB method (Grewe *et al.,* 1993). PCR was used to amplify a segment (~600 bp) of DNA from the 16S mitochondrial ribosomal RNA gene. DNA amplifications were performed in 50ì l volumes containing 5ìl of 10X PCR buffer, 3ìl of 25 mM MgCl2, 1ìl of 10 mM dNTPs (Promega, USA), 2.5ìl of 10pmol/ìl of each primer, 5ìl of template genomic DNA, 2ìl of 2ì/ìl Taq polymerase (Promega, USA) and 29ìl of ddH_2O. To amplify and sequence the 16SrDNA fragment, the primers 16S ar-L 5'-CGCCTGTTTATCAAAAACAT-3' and 16S br-H 5'-CCGGTCTGAACTCAGATCACGT-3' (Kocher *et al.,* 1989; Palumbi, 1996) were used.

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).

Multiple sequence alignment for forward reactions was carried out using CLUSTALX program version 1.81 (Thompson *et al.,* 1997), and subsequently aligned by eye. Modeltest 3.7 (Posada and Crandall, 1998) 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 (Swofford, 2002). Heuristic search NJ was performed using random sequence additions (n=10) and TBR branch swapping, bootstrap support values being 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) (Kluge and Farris, 1969; Farris, 1989) were computed in PAUP* version 4.0b10.

RESULTS

Results of the Modeltest 3.7 analyses showed that the substitution model of TrN+I+G (Tamura and Nei, 1993) provided the best fit to the data, selected by hierarchical likelihood ratio test (hLRT). Model parameters estimated were as follows: empirical base frequencies A = 0.3304, C = 0.2617, G = 1882 and T = 0.2196; nucleotide substitution rate [A-C] = 1.0000, [A-G] = 4.7424, [A-T] = 1.0000, [C-G] = 1.0000, [C-T] = 7.6066 and [G-T] = 1.0000; proportion of invariable sites (I) = 0.5067; gamma distribution shape parameter (á) = 0.6839.

All 562 nucleotide characters (381 constant; 30 parsimony-uninformative; 151 parsimony-informative) from 42 ingroup and 3 outgroup taxa were analyzed simultaneously, are presented at the species level for Leiognathidae in Figure 2. This resulted in one most-parsimonious tree with a length of 409 steps (CI = 0.6357; RI = 0.8867; RC = 0.5637; HI = 0.3643).

Gerreids and carangoids presently hypothesized to be close relatives of ponyfishes (Sparks *et al.,* 2005) were used as outgroup. In NJ tree, Leiognathidae was monophyletic with strong support by a bootstrap value of 100%. *Leiognathus equulus* formed the base of the others leiognathids supported by a moderate bootstrap value of 57%. *Leiognathus splendens* and *Leiognathus jonesi* formed a sister taxa to *Photopectoralis* species, with 88% bootstrap support. Trifurcation appeared among the group of *Photoplagios* spp., *Photoplagios stercorarius* and a group which comprising *Gazza* and *Secutor*. *Photoplagios* spp. except *Photoplagios stercorarius* formed a clade with 75% bootstrap support. *Gazza* formed a sister taxa to *Secutor* but with low bootstrap support. The NJ analysis

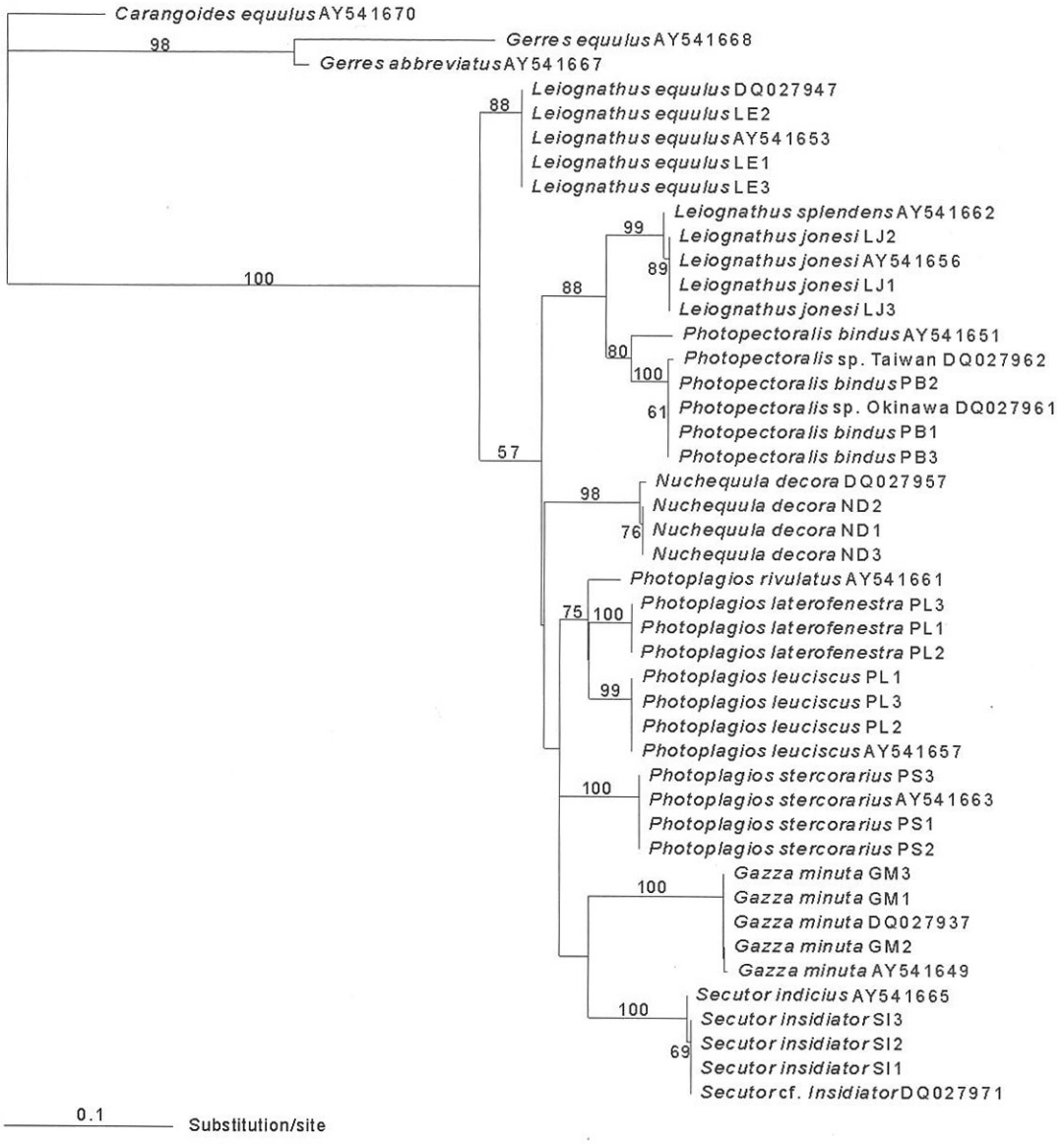

Figure 2. The neighbour-joining (NJ) tree of leiognathids based on the 16S mt-rDNA sequences. The tree included sequence data of Leiognathidae available from GenBank. Numbers at nodes represent bootstrap support. *Carangoides equulus*, *Gerres equulus* and *Gerres abbreviatus* were used as outgroups.

placed *Nuchequula* as sister taxa to the group of trifurcation. However, bootstrap support for this relationship was weak. The NJ tree suggested that the genus *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic.

DISCUSSION

In general the molecular phylogenetic positions of the fishes were in congruence with morphological delineation. The only exception was *Photoplagios stercoraroius* which was not placed in the group of *Photoplagios* spp. therefore formed a trifurcation in the NJ tree. *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. But all have clear lateral lining of the air bladder (Sparks and Dunlap 2004; *Sparks et al.,* 2005; Sparks 2006). In agreement with the features of the light organ system (LOS), it is probable that the

Photoplagios stercorarius place in the clade of *Photoplagios* spp. but in different branch. So, maybe *Photoplagios* have two distinct subclades.

Results of the molecular analysis also showed that the 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. Yet, 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. Hence the specimens from Okinawa and Taiwan were most probably *P. bindus*.

All members of leiognathids exhibit internal sexual dimorphism except *Leiognathus equulus*, which do not appear to be internal or external sexually dimorphism of the LOS (Sparks *et al.*, 2005) and formed the base of the others leiognathids in NJ tree. *Leiognathus splendens*, *Leiognathus jonesi* and *Nuchequula decora*, which just exhibit internally sexual dimorphism were placed among the sexually dimorphic leiognathids but they still have their own clade respectively. This result supports the suggestion that the light organ morphism is phylogenetically informative.

In conclusion, *Leiognathus* is paraphyletic, whereas *Gazza*, *Secutor*, *Photoplagios*, *Photopectoralis* and *Nuchequula* are monophyletic. Similar results were obtained by Ikejima *et al.* (2004), Sparks and Dunlap (2004) and *Sparks et al.* (2005) using a combination of different genetic markers. It is suggested that analysis of just 16S mitochondrial rDNA could be sufficient to resolve some of the outstanding taxonomic problems in the family Leiognathidae. A more robust morphological criterion, coupled with relevant molecular data should be applied to solve taxonomic uncertainties among leiognathids.

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