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Phylogeny and morphological delineation of leiognathids in the waters of Peninsular Malaysia

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Abstract — Taxonomic identification of leiognathids, which although are morphologically conservative fishes, is still problematic. In this study, a molecular phylogenetic approach was used together with morphological characterization to determine the taxonomy of leiognathids collected from Peninsular Malaysia waters. Phylogenetic relationships of 48 specimens from 18 morphospecies of leiognathids in this study together with 45 sequences from GenBank were inferred from 16S mitochondrial rRNA gene sequences. Neighbor-joining analysis showed that molecular phylogenetic positions of leiognathids were in congruence with morphological delineation at either genus- or species-level. The only exception at genus-level was the resurrection of *Aurigequula* from synonymy with *Leiognathus*. Members of both genera were recovered together as a monophyletic group in this study. At species-level, the exceptions were *Eubleekeria jonesi* and *Photopectoralis bindus*, both were morphologically identifiable. Molecular phylogenetics analysis placed *E. splendens* in the same group with *E. jonesi* while *P. panayensis* grouped together with *P. bindus.* A revision of the number of genera in the family Leiognathidae from Peninsular Malaysia is proposed from current 9 to 8, namely *Gazza*, *Leiognathus*, *Secutor*, *Photopectoralis*, *Nuchequula*, *Eubleekeria*, *Equulites* and *Karalla.* All genera of leiognathids in present study are monophyletic. Combination of these two approaches can resolve the taxonomic uncertainties of leiognathids.

Key words: Leiognathidae, Phylogeny, Taxonomy, 16S mitochondrial rRNA gene, Malaysia

Introduction

In Malaysia, leiognathids are named as "kekek"; probably the nickname is given by the chirping sound maked by leiognathids while they are still alive on board. In some parts of Malaysia, leiognathids are sold freshly or processed into popular fish crackers and salty dried fish, showing they are commercially important. Up to date, Leiognathidae comprises nine genera namely *Gazza*, *Leiognathus*, *Secutor*, *Photopectoralis*, *Nuchequula*, *Eubleekeria*, *Equulites*, *Aurigequula* and *Karalla* (Chakrabarty and Sparks 2008, Chakrabarty et al. 2008, Kimura et al. 2008c). However, *Karalla* is still not valid and synonymized with *Leiognathus* now (Eschmeyer 2010). Leiognathids are in need of taxonomic revision because diagnostic characters have been poorly defined, or described without a broad comparison to existing species, or alleged extant types do not match the corresponding original descriptions (Sparks 2006a). It is difficult to identify and diagnose leiognathids because they are morphologically conservative fishes across genera and may form species complex (Sparks et al. 2005). For these reasons, leiognathids taxonomy are plagued by numerous nomenclatural problems.

At present, several studies of leiognathids are carried out either on morphology or molecular work. The molecular phylogenetic analyses provide a better understanding of the relationship of leiognathids at the genus- and species-levels and it is a helpful tool to resolve some uncertainties of identification and also enhance the taxonomy revision (Ikejima et al. 2004, Sparks and Dunlap 2004, Sparks et al. 2005, Sparks 2006b, Chakrabarty and Sparks 2007, 2008).

There are 115 sequences from 31 species of leiognathids, inferred from 16S mitochondrial rRNA gene sequences, which have been deposited into GenBank. Ninety-two sequences (including 48 sequences in this study) were named by species name and others were unknown. However, GenBank just provided a platform to deposit species sequences, and is really lack of information to further study such as specimen description, morphometric parameters and figure. The aim of the current study was to analyze the molecular phylogeny and the congruence of molecular data with morphological descriptions of leiognathids present in

Malaysia waters.

Materials and Methods

Eighteen morphospecies of leiognathids were included in this study. Samples were collected from the waters of Pulau Tinggi, Pulau Perhentian, Tanjung Sepat and Kuala Kedah. Three individuals of each morphospecies were used in this study. 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, 2008c), Matsuura and Kimura (2005), Sparks (2006b), Chakrabarty and Sparks (2007) and Sparks and Chakrabarty (2007).

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 (Grewe et al. 1993). PCR was used to amplify a segment (~600 bp) of the 16S mitochondrial ribosomal RNA gene. DNA amplifications were performed in 50 μL volumes containing 5 μL of 10X PCR buffer, 3 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTPs (Promega, USA), 2.5 μL of 10 pmol/μL of each primer, 5 μL of template genomic DNA, 2 μL of 2 μ/μL Taq polymerase (Promega, USA) and 29 μL 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′) (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 Inc, USA).

Multiple sequence alignment for forward reactions was carried out using CLUSTALX 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 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) (Kluge and Farris 1969, Farris 1989) were computed in PAUP* version 4.0b10. Gerreids and carangoids were used as outgroups.

Results

A neighbour-joining tree of leiognathids based on 16S mt-rDNA sequences was generated (Fig. 1). Results of the Modeltest 3.7 analyses showed that the substitution model of GTR+I+G (Rodríguez et al. 1990) provided the best fit to the data, selected by Akaike information criterion (AIC). Model parameters estimated were as follows: empirical base frequencies A=0.3196, C=0.2581, G=0.1946 and T=0.2277; nucleotide substitution rate [A-C]=3.2438, [A–G]=10.7452 [A–T]=2.0790, [C–G]=0.3436, [C–T]=18.3052 and [G–T]=1.0000; proportion of invariable sites (I)=0.4765; gamma distribution shape parameter (α)=0.4964. All 578 nucleotide characters (372 constant; 31 parsimony-uninformative; 175 parsimony-informative) from 93 ingroup and 3 outgroup taxa were analyzed simultaneously. This resulted in one most-parsimonious tree with a length of 732 steps (CI=0.4467; RI=0.8615; RC=0.3849; HI=0.5533).

The NJ tree suggested that the family Leiognathidae (clade A) is monophyletic, strongly supported by a bootstrap value of 100%. Within Leiognathidae several clades were recovered: Clade B comprises *Aurigequula fasciata*, *A. longispinis*, *Leiognathus robustus* and *L. equulus* formed the base of the other clades (bootstrap value 57%), which do not appear to be sexually dimorphic regarding to the features of light organ system (LOS). Clade C comprises all the members of *Nuchequula* together with all members of *Gazza*, *Secutor*, *Photopectoralis*, *Equulites*, *Eubleekeria* and *Karalla*, which appear to be sexually dimorphic, however, three clades (D, E and I) only exhibit internal sexually dimorphism of LOS and others were sexually dimorphic for both internal and external of LOS.

Discussion

In general the molecular phylogenetic positions of leiognathids agreed well with morphological delineation. In the perspective of genera through this NJ tree, *Karalla* can be confirmed as a valid genus. The members of *Karalla* were placed in a distinct clade (clade E). Chakrabarty and Sparks

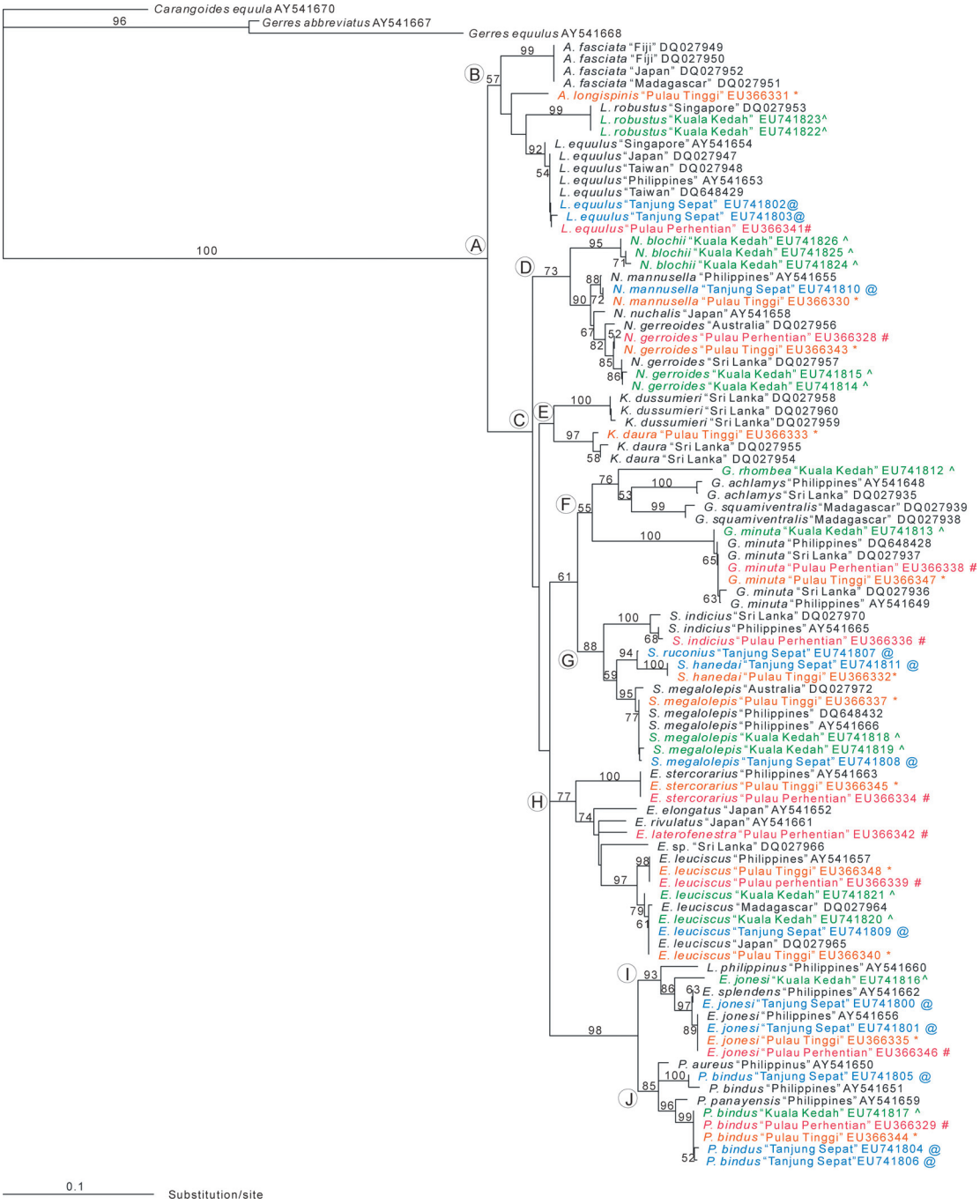

Fig. 1. Neighbour-joining tree of leiognathids based on 16S mt-rDNA sequences. The tree included sequence data of Leiognathidae available from GenBank. Numbers at nodes represent bootstrap support. Present study samples collected from different location were marked, * Pulau Tinggi, # Pulau Perhentian, @ Tanjung Sepat and ^ Kuala Kedah. *Carangoides equula*, *Gerres equulus* and *Gerres abbreviatus* were used as outgroups.

(2008) resurrected *Aurigequula* from synonyms of *Leiognathus*, and placed the two species, *Aurigequula fasciata* and *A. longispinis* in this genus. They have a series of yellow blotches arrayed horizontally along the flank and a markedly elongate second dorsal fin spine, however these features were not found in *L. robustus* and *L. equulus.* On the other hand, *L. striatus* have a series of yellow vertical bars along the flank but categorized in *Leiognathus* (Chakrabarty and Sparks 2008). These five species have large and oval body, and only appear as non-sexually dimorphic leiognathids, grouped in a clade and easily distinguished from other leiognathids. This result supports the suggestion that these five species maintain as genus *Leiognathus*.

Clade H comprises members of *Equulites*, there were two distinct subclades, in agreement with the features of translucent patch or stripe on flank (Seah et al. 2008). With

reference to Sparks (2006a) and Sparks and Chakrabarty (2007), there were three categories can be formed: species with translucent stripe (*E. stercorarius* and *E. moretoniensis*), species with expansive translucent patch either triangular, cornucopia, trapezoidal or bullet-shaped (*E. leuciscus*, *E. klunzingeri*, *E. rivulatus*, *E. laterofenestra* and *E. elongatus*), and species with translucent stripe and patch (*E. antongil*). Chakrabarty and Sparks (2008) suggested using genus name *Equulites* just for *E. leuciscus*, *E. klunzingeri* and *E. laterofenestra* with specific diagnoses. However, Kimura et al. (2008a) suggested *Equulites* is a senior synonym of *Photoplagios.* DNA sequences for all spesies in this category should be involved to study the complication of genus name usage.

Molecular phylogenetic positions of leiognathids were in congruence with morphological delineation except for *Eubleekeria jonesi* and *Photopectoralis bindus*. *Eubleekeria jonesi* and *P. bindus* specimens were morphologically identifiable. Kimura et al. (2005) confirmed that *L. philippinus* is a junior synonym of *E. splendens*. The specimen AY541660 mentioned as *L. philippinus* in GenBank should be identified as *Eubleekeria splendens* (see Kimura et al. 2008b). The specimen AY541656 is *E. jonesi* based on GenBank information, which has been mentioned incorrectly as *E. splendens* in Sparks et al. (2005). In clade I, the Philippines specimen AY541662 of *E. splendens* was placed in the group with *E. jonesi* and has 100% sequence similarity with the Malaysian specimen EU741800 of *E. jonesi.* So, these results suggested that the specimen AY541662 is probably *E. jonesi* and is required for a review. Molecular phylogenetics analysis grouped the Philippines specimen AY541659 of *Photopectoralis panayensis* together with *P. bindus. Photopectoralis bindus* had great DNA base variations even though they were morphologically identical (Seah et al. 2008). *Photopectoralis bindus* is easily distinguished from congeners by round body, orange blotch on spinous dorsal fin membrane and without a black line between anteroventral margin of orbit and lower jaw articulation (Woodland et al. 2001, Kimura et al. 2003). Thus the specimen from the Philippines was most probably *P. bindus* and is required for a review.

The Malaysian specimen EU366336 was incorrectly assigned by the authors to *Secutor insidiator* (Seah et al. 2008). After detailed examination, the pecimen EU366336 was determined to represent *S. indicius*. A misidentification was due to the quality of the specimen and almost similar descriptions available in literature references. Further investigation showed that some stripes in these morphotype specimens (shown as Fig. 4b, Seah et al. 2008) are still discernible but in faint condition. If those faint stripes were highlighted, the specimens showed the similarity to *S. indicius* (shown as Fig. 4a, Seah et al. 2008). An illustration in Woodland et al. (2001) further validates both morphotype specimen were *S. indicius* with the presece of a black line concentrated by melanophores extending from the posterior part of the pectoral fin axilla to the posterior part of the pelvic fin. Thus it is probable that the *S.* cf. *insidiator* DQ027971 also is one of this species.

Results of this study showed that Leiognathidae from Peninsular Malaysia comprises eight genera namely *Gazza*, *Leiognathus*, *Secutor*, *Photopectoralis*, *Nuchequula*, *Eubleekeria*, *Equulites* and *Karalla*. All genera of Leiognathidae are monophyletic. In general, from previous studies, it is suggested that a more complete gathering of leiognathids species sequences is needed to examine the status of genera especially for those valid species. Besides, relevant molecular data analyses enhanced the taxonomic work processes although morphological characteristic identification has higher priority. Combination of these two approaches resolves the taxonomic uncertainties of leiognathids.

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