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Molecular systematics and phylogenetic analysis of the Asian endemic freshwater sleepers (Gobiiformes: Odontobutidae)

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ABSTRACT

The Odontobutidae is a group of freshwater sleepers endemic to East and Southeast Asia. The composition of the Odontobutidae is controversial and the systematics position of some species (e.g. *Philypnus chalmersi*) remains unknown. Phylogenetic relationship among the odontobutids has never been really tested due to the lack of informative morphological characters, and that molecular data have not been collected in many species. Here, we sampled 41 specimens, representing all known genera of the Odontobutidae except the Laotian genus *Terateleotris*, in addition to a disputable odontobutid species, *Philypnus chalmersi* and 14 outgroups (six families). We collected sequence data of 4434 single-copy nuclear coding loci using gene capture and Illumina sequencing. A robust phylogeny of the odontobutids and outgroups was built, confirming that the Odontobutidae is monophyletic and sister to the Rhyacichthyidae. We verified that *Neodontobutis*, *Sineleotris* and *Philypnus chalmersi* are members of the Odontobutidae based on the resulting phylogeny as well as patterns of pectoral girdle examined by X-ray microtomography. We proposed a new genus *Microdous* for *Philypnus chalmersi* based on the new morphological and molecular evidences. The family of the Odontobutidae can be divided into two clades: *Microdous* (=*Philypnus*) sister to a group consisting of *Micropercops* and *Sineleotris*, and *Odontobutis* sister to a group unifying *Perccottus* and *Neodontobutis*. Divergence time among the odontobutids was estimated based on 100 most clock-like loci and three fossil calibration points using BEAST. Ancestral range of the family was reconstructed using Reconstruct Ancestral States in Phylogenies (RASP) and BioGeoBEARS. The results suggest that the common ancestor of the odontobutids originated around 30.8 Ma (20.7–42.0 Ma, 95% HPDs) in South China. Orogeny, climatic change and river capture might account for diversification and current distribution of the odontobutids.

1. Introduction

The Odontobutidae, an early branching lineage of the Gobiiformes (Thacker, 2009; Thacker and Hardman, 2005), comprises about 15–22 species in six genera (*Terateleotris*, *Sineleotris*, *Micropercops*, *Perccottus*, *Neodontobutis*, *Odontobutis*) (Froese and Pauly, 2017; Iwata, 2011). Odontobutids are distinct from the majority of gobiiforms; they are larger-bodied ranging from 3 to 20 cm in size, less morphologically reduced, and restricted to freshwater (Iwata et al., 1987, 1988a,b; Iwata et al., 2001). Some species of the odontobutids have commercial value (e.g. *O. potamophila*, *O. sinensis* and *P. glenii*), whereas some others are vulnerable or endangered (e.g. *N. hainanensis*, *O. yaluensis* and *S. saccharae*) (Wu, 2008).

Many questions about the odontobutids are understudied, such as composition of the Odontobutidae and phylogenetic relationships among the odontobutids (Iwata, 2011). This lack of systematic knowledge has hampered us from understanding the evolution of morphological specializations in the odontobutids. The odontobutids are distributed in East and Southeast Asia, including Japan, Russia, Korea, eastern China, Laos and Vietnam. Knowledge about the systematics and biogeography of the odontobutids could help us to investigate the origin of freshwater fish fauna in East Asia in general.

Hoese and Gill (1993) established the family Odontobutidae with three genera (*Micropercops*, *Odontobutis* and *Perccottus*). They used eight characters to describe this family, involving infraorbital bones, sclerotic bones, scapula, middle radial of first pterygiophore of second dorsal, dorsal procurrent caudal cartilage, the arrangement of papillae, pterygiophore formula of first dorsal and truncated cteni, but they were

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unable to provide evidence for monophyly of the Odontobutidae. A possible synapomorphy of the Odontobutidae involves the condition of the pectoral girdle, in which the scapula is large, excluding proximal radial from contacting with cleithrum, but this character has only been examined in *Micropercops* and *Odontobutis* (Hoese and Gill, 1993; Nelson, 2006; Shen, 2005; Wu, 2008). A series of morphological studies were conducted subsequently (Ahnelt and Göschl, 2004; Iwata et al., 2001; Springer and Johnson, 2004). Nevertheless, monophyly of the Odontobutidae is still disputable, probably due to the lack of synapomorphy of this family and uncertain status of some recently proposed members of the family.

Neodontobutis, *Sineleotris*, *Philypnus* and *Terateleotris* were added to the family lately by Chen et al. (2002) and Nelson (2006). *Neodontobutis* was included based on simple longitudinal infraorbital papillae pattern (Chen et al., 2002), which, however, is not an established synapomorphy of the odontobutids. Similarly, there is no solid evidence to assign *Terateleotris* to the Odontobutidae (Nelson, 2006; Shibukawa et al., 2001). *Philypnus chalmersi* sometimes was thought as a species of *Perccottus* (Fowler, 1962; Zheng, 1981), or a species of *Sineleotris* (Chen et al., 2002), both of which are genera assigned to the Odontobutidae. Nevertheless, no solid morphological evidences were provided for adding the new species members to the family of Odontobutidae, and their status are unstable (Iwata, 2011; Wu, 2008).

Similarly, molecular analyses on the odontobutids are scarce. Mostly only mitochondrial loci and a few taxa were sampled, and the conclusions are inconsistent among studies (Akihito et al., 2000; Jun et al., 2016; Thacker, 2009; Thacker et al., 2015; Zang et al., 2016). The monophyly of the Odontobutidae is generally supported by molecular data, but they often only involved two or three odontobutids and phylogenetic interrelationships within the Odontobutidae are still contentious. For example, molecular phylogeny based on four mtDNA genes showed that *Odontobutis* and *Perccottus* were closely related to each other (Thacker and Hardman, 2005), but in mitochondrial genome analyses, *Micropercops* and *Perccottus* were grouped together (Jun et al., 2016; Zang et al., 2016).

In the present study, we reconstructed the first phylogeny of the Odontobutidae with genomic data and a comprehensive taxon sampling including 41 individuals of eight representative odontobutid species from six genera as well as 16 outgroups. We collected sequence data from 4434 nuclear loci using targeted gene capture and next-generation sequencing (Li et al., 2013; Jiang et al., 2017). Our objectives are: (1) to validate whether the newly added species (i.e. *P. chalmersi* and *N. hainanensis*) indeed belong to the Odontobutidae using both molecular data as well as microtomography of pectoral girdle; (2) to infer phylogenetic relationships within the family; (3) to estimate divergence time between the odontobutid species and test hypotheses about their evolutionary history and biogeography.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Fin clips or muscle tissues were obtained from eight species (41 individuals) of the Odontobutidae (Table 1). Each species was represented by three to six individuals from different regions. All genera of the family were sampled except for *Terateleotris*. The analysis also covered 14 outgroup taxa (six families) including *Rhyacichthys aspro* (Rhyacichthyidae), *Butis koilomatodon*, *Kribia nana*, *Oxyeleotris marmorata* and *Bostrychus sinensis* (Butidae), *Eleotris acanthopoma*, *E. oxycephala*, *Gobiomorus dormitor* and *Dormitator maculatus* (Eleotridae), *Milyeringa veritas* and *Typhleotris pauliani* (Milyeringidae), *Kurtus gulliveri* (Kurtidae), *Sphaeramia orbicularis* and *Pterapogon kauderni* (Apogonidae). Genomic DNA was extracted from ethanol-preserved tissue using an Ezup Column Animal Genomic DNA Purification Kit (Sangon, Shanghai, China). The purified DNA was quantified with a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) and visualized by agarose gel electrophoresis.

2.2. Bait design

A suite of 4434 single-copy nuclear coding sequence (CDS) makers were adopted from Jiang et al. (2017). Regions with abnormally high read coverage in a pilot experiment were masked from redesigning the baits (Jiang et al., 2017). Length of these markers was between 120 bp and 5,161 bp. Biotinylated RNA baits of 120 bp with 2× tiling was synthesized based on sequences of *Rhinogobius giurinus* (Gobiiformes) (MYcroarray, Ann Arbor, Michigan; cat#, 150901-Li-Goby). When sequences of *R. giurinus* were not available for some target loci, sequences of other gobiiforms were used. The sequences of target loci and baits are listed in Supplemental Materials.

2.3. Library preparation, gene capture, pooling and sequencing

Library preparation and gene capture were carried following Meyer and Kircher (2010) with modifications (Li et al., 2013) to accommodate capturing target loci of divergent species. The purified genomic DNA was sheared to around 250 bp using a Covaris M220 Focused-ultrasonicator (Woburn, Massachusetts, U.S.A.). A total of 300 ng sheared DNA was used for library preparation. To facilitate multiplex sequencing, each sample was labeled with an 8 bp index on the P7 adapter. Enriched and indexed products were pooled in equimolar ratios for sequencing on an Illumina HiSeq X10 lane at Annoroad (Beijing, China).

2.4. Data assembly

Reads from each sample were parsed according to their 8 bp index. Data assembly was performed following the description in Yuan et al. (2016). The output amino acid sequences were aligned in batch using Clustal Omega (Sievers et al., 2011). The aligned AA sequences were translated back to DNA alignment for subsequent analyses. Pairwise distance of all loci was calculated and loci with abnormal high distance values were realigned by eye or discarded if not correctable.

2.5. Phylogenetic analyses

The aligned DNA sequences were used for reconstructing phylogeny. Concatenated maximum likelihood trees were reconstructed under GTRGAMMA model in RAxML v8.0.0 (Stamatakis, 2014) with 1000 bootstrap replicates. Data were partitioned by codon position as well as by a partitioning scheme identified with PartitionFinder v2.0 (Lanfear et al., 2012). The best partitioning scheme was selected with BIC criterion, GTRGAMMA model and rcluster algorithm (Lanfear et al., 2014).

To infer species tree, each individual gene tree was reconstructed with RAxML v8.0.0 under the GTRGAMMA model. Then, ASTRAL 4.10.6 (Mirarab et al., 2014) was used to generate a species tree from all gene trees. Two options "-q" "-t 2" was chosen to estimate the quartet support. ASTRAL, a coalescent-based analysis, can run on large datasets and it is often more accurate than concatenation using maximum likelihood, if incomplete lineage sorting levels are high (Mirarab et al., 2016; Mirarab and Warnow, 2015). The resulting trees were visualized in FigTree v.1.4.0 (Rambaut, 2013).

2.6. X-ray microtomography

Specimens of *P. chalmersi*, *M. swinhonis*, *S. saccharae*, *P. glenii*, *N. hainanensis* and *O. yaluensis* were scanned using MicroCT Skyscan 1176 (Bruker, Belgium) with 45 kV tube voltage, 0.3-degree rotation step, and 8.7 μm pixel resolution. The cross-sections of each specimen were reconstructed. The three-dimensional renderings were created, visualized, and manipulated in the VG Studio Max v2.1 (Heidelberg,