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

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,

Table 1
Taxon sampling, summary of the sequencing results of 41 individuals of the Odontobutidae and 14 outgroup taxa.

Voucher number	Species	Collection site	No. raw reads[a]	No. filtered reads[b]	No. unique reads[c]	No. captured loci[d]	Abbreviation
CL1227_2	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	4,217,814	4,217,808	1,960,646	2885	PR1227_2
CL1227_3	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	4,526,264	4,526,260	2,154,784	2892	PR1227_3
CL1228	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	4,862,956	4,862,948	2,612,778	2754	PR1228
CL1279_1	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	3,663,098	3,663,088	2,127,488	2768	PR1279_1
CL1279_3	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	4,172,640	4,172,632	2,463,972	2800	PR1279_3
CL1279_4	*Neodontobutis hainanensis*	Pearl River, Yangjiang, Guangdong	3,981,800	3,981,786	2,448,232	2859	PR1279_4
CL632_1	*Perccottus glenii*	Songhua River, Harbin, Heilongjiang	8,190,024	8,189,942	3,320,210	3375	SH632_1
CL632_2	*Perccottus glenii*	Songhua River, Harbin, Heilongjiang	5,009,738	5,009,722	1,399,952	3171	SH632_2
CL1149_9	*Perccottus glenii*	Liaohe River, Shenyang, Liaoning	4,114,778	4,114,770	1,330,010	3165	LH1149_9
CL1149_10	*Perccottus glenii*	Liaohe River, Shenyang, Liaoning	11,627,320	11,627,252	5,514,880	3582	LH1149_10
CL1149_11	*Perccottus glenii*	Liaohe River, Shenyang, Liaoning	3,545,264	3,545,256	1,768,262	3136	LH1149_11
CL353_1	*Odontobutis sinensis*	Yangtze River, Dangyang, Hubei	12,905,792	12,905,722	5,476,860	3593	YZ353_1
CL808_11	*Odontobutis sinensis*	Pearl River, Guilin, Guangxi	5,731,536	5,731,528	3,479,838	3349	PR808_11
CL812_2	*Odontobutis sinensis*	Pearl River, Quanzhou, Guangxi	9,093,744	9,093,732	5,085,422	3529	PR812_2
CL817_4	*Odontobutis sinensis*	Yangtze River, Xiangtan, Hunan	9,391,882	9,391,872	5,531,844	3516	YZ817_4
CL821_2	*Odontobutis sinensis*	Yangtze River, Dongting Lake, Hunan	8,487,940	8,487,934	4,899,606	3507	YZ821_2
CL850_8	*Odontobutis yaluensis*	Yalujiang River, Liaoning	11,357,646	11,357,578	6,039,054	3493	YL850_8
CL1139_2	*Odontobutis yaluensis*	Yalujiang River, Dandong, Liaoning	3,178,170	3,178,164	1,523,790	3084	YL1139_2
CL1166_1	*Odontobutis yaluensis*	Yalujiang River, Dandong, Liaoning	10,439,214	10,439,206	5,545,976	3637	YL1166_1
CL1275_1	*Odontobutis yaluensis*	Yalujiang River Dandong, Liaoning	4,012,832	4,012,830	2,266,570	2833	YL1275_1
CL1275_4	*Odontobutis yaluensis*	Yalujiang River Dandong, Liaoning	3,418,018	3,418,014	1,787,328	2768	YL1275_4
CL378_8	*Odontobutis potamophila*	Yangtze River, Xuancheng, Anhui	11,142,666	11,142,658	4,801,920	3607	YZ378_8
CL382_9	*Odontobutis potamophila*	Yangtze River, Chaohu, Anhui	3,614,870	3,614,862	1,084,502	3082	YZ382_9
CL399_4	*Odontobutis potamophila*	Yangtze River, Luan, Anhui	10,479,902	10,479,760	3,811,824	3259	YZ399_4
CL421_3	*Odontobutis potamophila*	Yangtze River, Gaoyou, Jiangsu	10,943,612	10,943,552	6,082,402	3555	YZ421_3
CL478_8	*Odontobutis potamophila*	Yangtze River, Suzhou, Jiangsu	2,680,740	2,680,736	699,244	2897	YZ478_8
CL1224_3	*Sineleotris saccharae*	Pearl River, Enping, Guangdong	8,012,214	8,012,194	4,367,900	3606	PR1224_3
CL1224_4	*Sineleotris saccharae*	Pearl River, Enping, Guangdong	11,258,706	11,258,658	6,709,510	3598	PR1224_4
CL1224_5	*Sineleotris saccharae*	Pearl River, Enping, Guangdong	4,653,146	4,653,126	3,365,334	3284	PR1224_5
CL1229_1	*Sineleotris saccharae*	Pearl River, Huizhou, Guangdong	4,386,656	4,386,646	2,621,390	2743	PR1229_1
CL1229_2	*Sineleotris saccharae*	Pearl River, Huizhou, Guangdong	5,144,070	5,144,056	2,722,114	2850	PR1229_2
CL1229_3	*Sineleotris saccharae*	Pearl River, Huizhou, Guangdong	3,344,676	3,344,670	2,210,940	2836	PR1229_3
CL8_3	*Micropercops swinhonis*	Yangtze River, Langxi, Anhui	10,404,662	10,404,530	4,715,992	3373	YZ8_3
CL367_5	*Micropercops swinhonis*	Minjiang River, Huzhou, Zhejiang	9,194,792	9,194,746	3,115,670	3302	MJ367_5
CL425_10	*Micropercops swinhonis*	Yangtze River, Jingjiang, Jiangsu	3,762,192	3,762,180	932,028	2985	YZ425_10
CL442_4	*Micropercops swinhonis*	Yangtze River, Jingzhou, Hubei	369,254	369,254	230,056	1852	YZ442_4
CL816_5	*Micropercops swinhonis*	Yangtze River, Shaoyang, Hunan	7,071,504	7,071,476	3,522,092	3404	YZ816_5
CL1281_1	*Micropercops swinhonis*	Yangtze River, Guiyang, Guizhou	4,051,948	4,051,936	2,490,116	2938	YZ1281_1
CL886_1	*Microdous[e] chalmersi*	Pearl River, Baishou, Guangxi	10,717,450	10,717,402	6,790,610	3735	PR886_1
CL886_2	*Microdous chalmersi*	Pearl River, Baishou, Guangxi	10,138,370	10,138,292	6,514,558	3677	PR886_2
CL929	*Microdous chalmersi*	Wuzhishan, Hainan	4,140,720	4,140,708	3,106,894	3622	HN929
Outgroup							
CL304	*Gobiomorus dormitor*	–	8,468,862	8,365,736	7,351,680	3506	304
USNM410571	*Eleotris acanthopoma*	–	8,574,974	8,344,860	6,321,392	3565	410,571
–	*Dormitator maculatus*	–	7,381,820	7,222,658	6,877,706	3265	–
CL1209_1	*Eleotris oxycephala*	Fuchunjiang River, Tonglu, Zhejiang	2,337,812	2,337,808	1,610,440	3648	1209_1
CL553	*Butis koilomatodon*	–	7,762,040	7,582,582	5,786,312	3504	553
CL305	*Kribia nana*	–	5,895,928	5,815,014	4,682,068	3042	305
CL24_1	*Oxyeleotris marmoratus*	–	6,473,574	6,341,852	4,986,350	3280	24_1
CL1203	*Oxyeleotris marmoratus*	Yangjiang, Guangdong	4,572,004	4,571,994	3,320,842	3469	1203
CL1298_1	*Bostrychus sinensis*	Zhanjiang, Guangdong	3,823,118	3,823,104	1,335,668	2893	1298_1
CL1298_2	*Bostrychus sinensis*	Zhanjiang, Guangdong	3,942,262	3,942,252	1,854,020	2856	1298_2
CL303	*Rhyacichthys aspro*	–	7,327,646	7,222,068	6,099,778	3623	303
AMNH245649	*Typhleotris pauliani*	–	5,056,802	4,924,896	4,257,202	3408	245,649
LSUMZ13636	*Milyeringa veritas*	–	7,609,098	7,460,506	4,712,088	3624	13,636
CL116	*Kurtus gulliveri*	–	8,225,564	8,007,234	5,966,774	4078	116
CL349	*Sphaeramia orbicularis*	–	11,081,578	10,918,472	6,895,016	3654	349
CL348	*Pterapogon kauderni*	–	11,810,784	11,656,832	8,369,084	3634	348
In total	–	–	759,351,158	755,736,900	188,247	5182	–
On average	–	–	6,697,973	6,666,269	3,843,140	3262	–

"–" stands for missing data.
[a] The number of the raw reads.
[b] The number of reads after trimming the adapter and low quality reads.
[c] The number of reads after removing PCR duplicates.
[d] The number of captured loci.
[e] A new genus (=*Philypnus*).
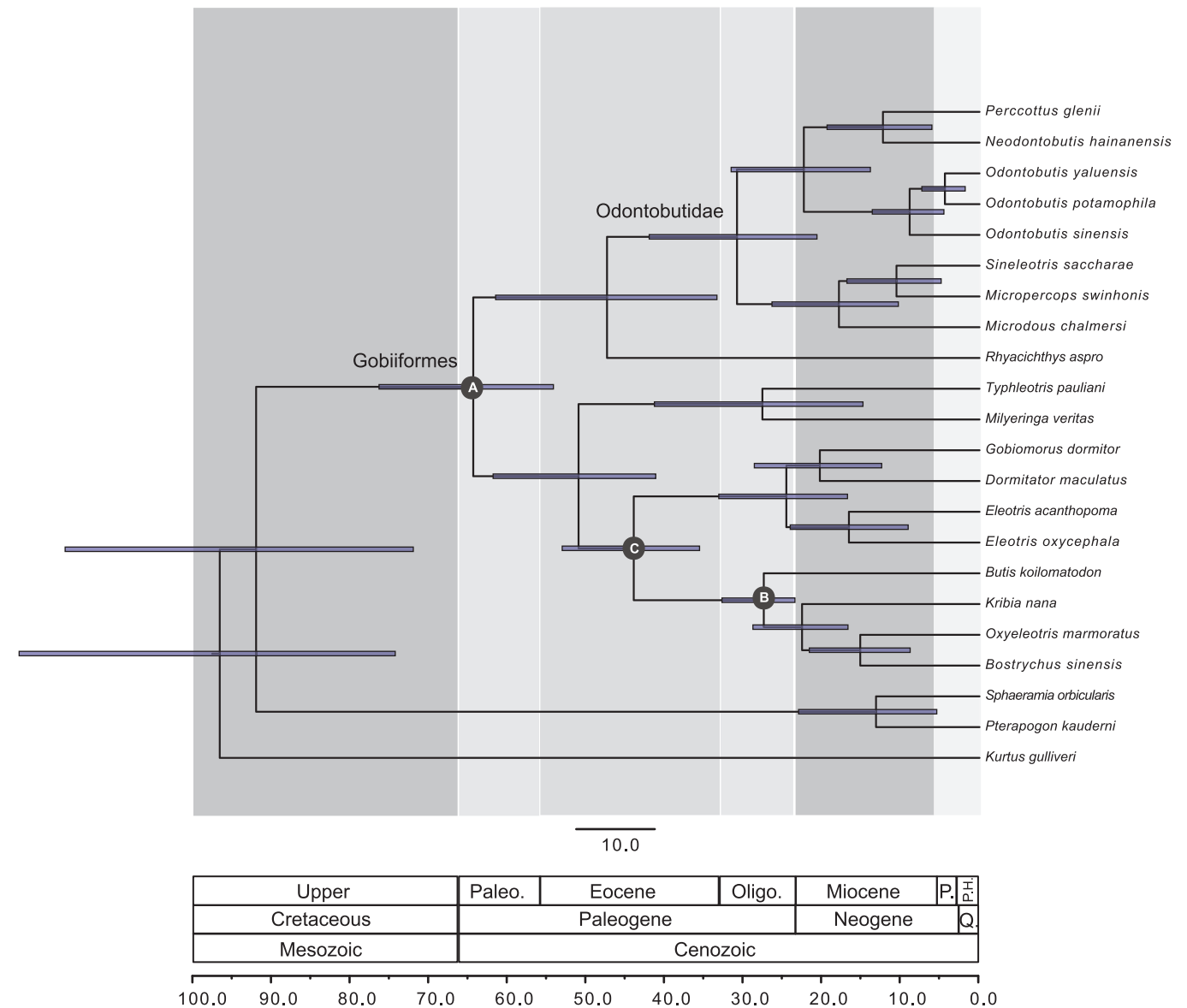
Fig. 1. Divergence time estimation of the Odontobutidae derived from BEAST analysis based on 100 most clock-like loci. Shaded bars on the nodes represent 95% highest posterior credible age intervals for each node. Black dots with letters (A, B and C) represent fossil calibration points.

Germany). By adjusting the gray value threshold, the image of bones remained while the soft tissues were virtually invisible (He et al., 2013).

2.7. Estimation of divergence time

In order to calibrate the divergence time among the odontobutids, 100 most clock-like loci were selected from those loci that had no missing data, including all 22 species (8 ingroups and 14 outgroups). To select the 100 most clock-like loci, individual gene trees were re-constructed under a strict-clock and a relaxed-clock model. Then, the log likelihood ratio between the relaxed-clock and the strict-clock model was used to rank the loci. BEAUti v2.3.2 (Drummond and Rambaut, 2007) was used to create BEAST (Bouckaert et al., 2014) input files written in XML. Data were split into three codon positions (1 + 2 + 3). Site models were unlinked across partitions, while clock models and trees were linked. The substitution model was GTRGAMMA with 1.0 substitution rate and 4 Gamma category count. Clock model was relaxed clock log normal and tree model was birth-death. The RAxML tree based on the concatenated alignment was calibrated with fossil dates (listed in following paragraph) using a penalized likelihood

method in R package ape 4.1 (Paradis et al., 2004), and the resulting tree was used as a starting tree for the BEAST analysis to avoid potential failure of the BEAST run when using a random starting tree. The weight of four parameters: Birth Death Subtree Slide, Narrow, Wide and Wilson-Balding were set as zero to fix the tree topology in the BEAST run. The BEAST analysis was run twice with 60,000,000 generation of MCMC, and parameter sampling every 1000 generations. The MCMC log files were examined in Tracer v.1.6 (Rambaut et al., 2014) to assess convergence and calculate effective sample sizes (ESS) of various parameters. Tree files from two runs were combined by LogCombiner v.2.3.2 with 20% Burn-in (Drummond and Rambaut, 2007). TreeAn-notator v.1.8.1 (Drummond and Rambaut, 2007) was used to make a maximum clade-credibility (MCC) tree with means and 95% highest posterior density of divergence times. The number of burn-in was set at 20%. FigTree v.1.4.0 was used to view the final trees.

In the divergence time estimation, three fossil calibration points were used for setting priors. Due to the lack of fossil record of odon-tobutids, nodes splitting related outgroups were chosen as fossil cali-bration points. †*Carlomonnius quasigobius* was described as *incertae sedis* within Gobiiformes and the oldest member of the order according

to articulated skeletal remains (Bannikov and Carnevale, 2016). Placement of †*Carlomonnius quasigobius* within the Gobiiformes was based on the absence of parietals and basisphenoid, the absence of uroneurals, supraneurals and lateral line, the presence of a large subquadrangular saccular otolith, the infraorbital series represented only by the lachrymal, the presence of a suspensorial interspace, caudal skeleton with reduced parhypural and hypurals 1 + 2 fused to 3 + 4, the presence of cleithral notch. †*Carlomonnius quasigobius* was found in Monte Bolca in northern Italy with an estimated stratum age at Late Ypresian (47.8–56.0 Ma) (Bannikov and Carnevale, 2016). A second fossil, †*Lepidocottus aries* was related to the extant Butidae and as a sister to *Kribia* based on characters: decreasing number of caudal fin rays, secondary cycloid predorsal scales, a rectangular shape of otolith and an anteriorly extended sulcus, a pterygiophore arrangement pattern of 2211, and an infraorbital bone (Gierl et al., 2013). The fossil of †*Lepidocottus aries* was found in Aix-en-provence in France and its stratum age was estimated at latest Oligocene epoch (23.03–27.82 Ma) (Gierl et al., 2013). The third fossil, †*Paralates chapelcorneri* was considered as a member of the Gobiiformes because of the following features: suspensorium fenestrae are large; parietals are absent; hypurals 1 and 2 are fused; hypurals 3 and 4 are fused and to the urostyle. Only the extant Eleotrid *Gobiomorphus* reveals six branchiostegals and a similar pterygiophore formula (3-212110 vs. 3-2121100) and vertebrae count (12 + 19 vs. 12 + 17–19) as seen in †*Paralates*. Due to the moderate preservation of the fossils, most other counts are uncertain. So the relationship between the fossil *Gobiomorphus* sp. and †*Paralates* is uncertain. The fossil of †*Paralates* was found in Isle of Wight (UK) with an estimated stratum age at Priabonian (33.9–37.8 Ma) (Gierl and Reichenbacher, 2017).

A log-normal distribution with hard minimum age, 47.8 Ma and a 95% soft maximum age, 122 Ma (mean, 3.48; standard deviation, 0.5; offset, 47.8) were applied to the ancestral node of Gobiiformes (Fig. 1A). The hard minimum age, 47.8 Ma was based on the minimum estimated age of the oldest gobiiform fossil, †*Carlomonnius quasigobius*, whereas the soft maximum, 122 Ma, was based on an oldest estimate of the root of the gobiiforms using mitochondrial data (Chakrabarty et al., 2012). The ancestor node of *Kribia* and *Butis* (Fig. 1B) was constrained with a hard minimum age, 23.03 Ma based on the minimum age estimation of †*Lepidocottus aries* and a 95% soft maximum age, 37.8 Ma based on the maximum age estimate of †*Paralates* (mean, 1.38; standard deviation, 0.8; offset, 23.03). The common ancestor of Eleotridae and Butidae (Fig. 1C) was constrained with a hard minimum age, 33.9 Ma based on †*Paralates* and a 95% soft maximum age, 56 Ma based on the maximum age estimate of †*Carlomonnius quasigobius* (mean, 1.78; standard deviation, 0.8; offset, 33.9).

2.8. Reconstruction of ancestral distribution of the Odontobutidae

Models implemented in RASP v3.1 (Ree and Smith, 2008; Yu et al., 2015) were applied to reconstruct ancestral distribution of the odontobutids. The number of maximum distribution was constrained as two. BioGeoBEARS with three models: DEC, DIVALIKE, and BAYAREALIKE was used to infer probabilistic historical biogeography (Matzke, 2013) as a comparison to the results from RASP analyses. The Models were evaluated by AIC scores. The calibrated time tree from the above BEAST analyses was used as input tree with outgroups discarded. Nine distribution areas were implemented in the analyses based on the major river systems and distribution areas of the odontobutids (Table S1): A. the Korea Peninsula; B. the Amur River, the Songhua River and Russian Far East; C. the Liaohe River and the Yalujiang River; D. the Yellow River; E. the Yangtze River and the Huaihe River; F. the Minjiang river, independent rivers in Fujian and Zhejiang provinces; G. the Pearl River and independent rivers in Hainan Island; H. the Indo-China Peninsula; I. Japan (Iwata, 2011; Li, 1981; Wu, 2008). To estimate the effect of unsampled species of the odontobutids on the biogeographic analyses, the terminal taxa of the species tree were collapsed as genera and distributions of the whole genus were used for biogeographic analyses as a

comparison, based on the observation that all genera are monophyletic (Table S1).

3. Results

3.1. Statistics of sequencing result and reads assembly

Illumina sequencing generated 381,784,486 raw reads that is 6,697,973 reads per sample on average (57 samples in total). After adapter trimming and excluding low quality reads, there were 6,666,269 reads per sample. The smallest number of reads per sample was 369,254 (*M. swinhonis*, CL442_4) and the largest was 12,905,722 (*O. sinensis*, CL353_1) (Table 1). When PCR duplicates were removed, 3,843,141 unique reads were left for each sample on average, accounting for more than 57% of the original reads. All 4434 loci have sequences collected in at least one species. Loci with large average pairwise distances were checked by eye. After discarding loci that cannot be aligned confidently, 4397 loci were left, with an average of 3262 loci per sample. *Micropercops swinhonis* (CL442_4) has the fewest number of loci (1852) and *K. gulliveri* (CL116) has the most (4078). The total length concatenating all loci was 1,190,397 bp with 271 bp per locus on average. There were on average 25.6% missing data in the concatenated alignment. The raw data with adapters and low quality reads trimmed are lodged in GenBank with accession number SRP127338. The aligned sequences can be found in Supplementary Materials.

3.2. Phylogenetic analyses

The ML tree based on best partitioning scheme identified by PartitionFinder is consistent with the ML analysis according to codon position, and all inter-specific nodes have a bootstrap support of 100 (Figs. 2 and S1). The species tree reconstructed using ASTRAL is also consistent with the ML trees (Fig. 3). Bootstrap values of the species tree are 100 for all nodes. The normalized quartet score of ASTRAL analysis is 0.647.

In the resulting phylogeny, the Odontobutidae is a monophyletic family with *R. aspro* as the sister taxon. All odontobutid species are also monophyletic. The odontobutids are grouped into two clades: *P. chalmersi* sister to the clade consisting of *Micropercops* and *Sineleotris*, and *Odontobutis* (represented by *O. sinensis, O. potamophila* and *O. yaluensis*) sister to the clade unifying *Perccottus* and *Neodontobutis*.

3.3. Skeleton of pectoral girdle

The skeleton of pectoral girdle was investigated using X-ray microtomography. The scapula is fully ossified and well developed, which excludes the upper proximal radial from contact with the cleithrum in all six species examined (*P. chalmersi, M. swinhonis, S. saccharae, P. glenii, N. hainanensis* and *O. yaluensis*; Fig. 4).

3.4. Fossil calibration

Both independent BEAST runs from random starting seeds reached stationarity. ESS values of all parameters were greater than 500. The mean divergence times and their 95% higher posterior densities (HPDs) intervals are shown in Fig. 1. The divergence time between the Rhyacichthyidae and the Odontobutidae was inferred as in the Eocene epoch (47.3 Ma, 33.4–61.5 Ma, 95% HPDs). The most recent common ancestor (MRCA) of odontobutids was estimated at 30.8 Ma (20.7–41.9 Ma, 95% HPDs) during the Oligocene. The common ancestor of the clade composing *Odontobutis, Perccottus* and *Neodontobutis* was dated to the early Miocene (22.3 Ma, 13.9–31.6 Ma, 95% HPDs). The divergence time between *Perccottus* and *Neodontobutis* was in the Miocene (12.3 Ma, 6.1–19.4 Ma, 95% HPDs). The clade composed of *P. chalmersi, Micropercops* and *Sineleotris* was dated to the Miocene (17.9 Ma,
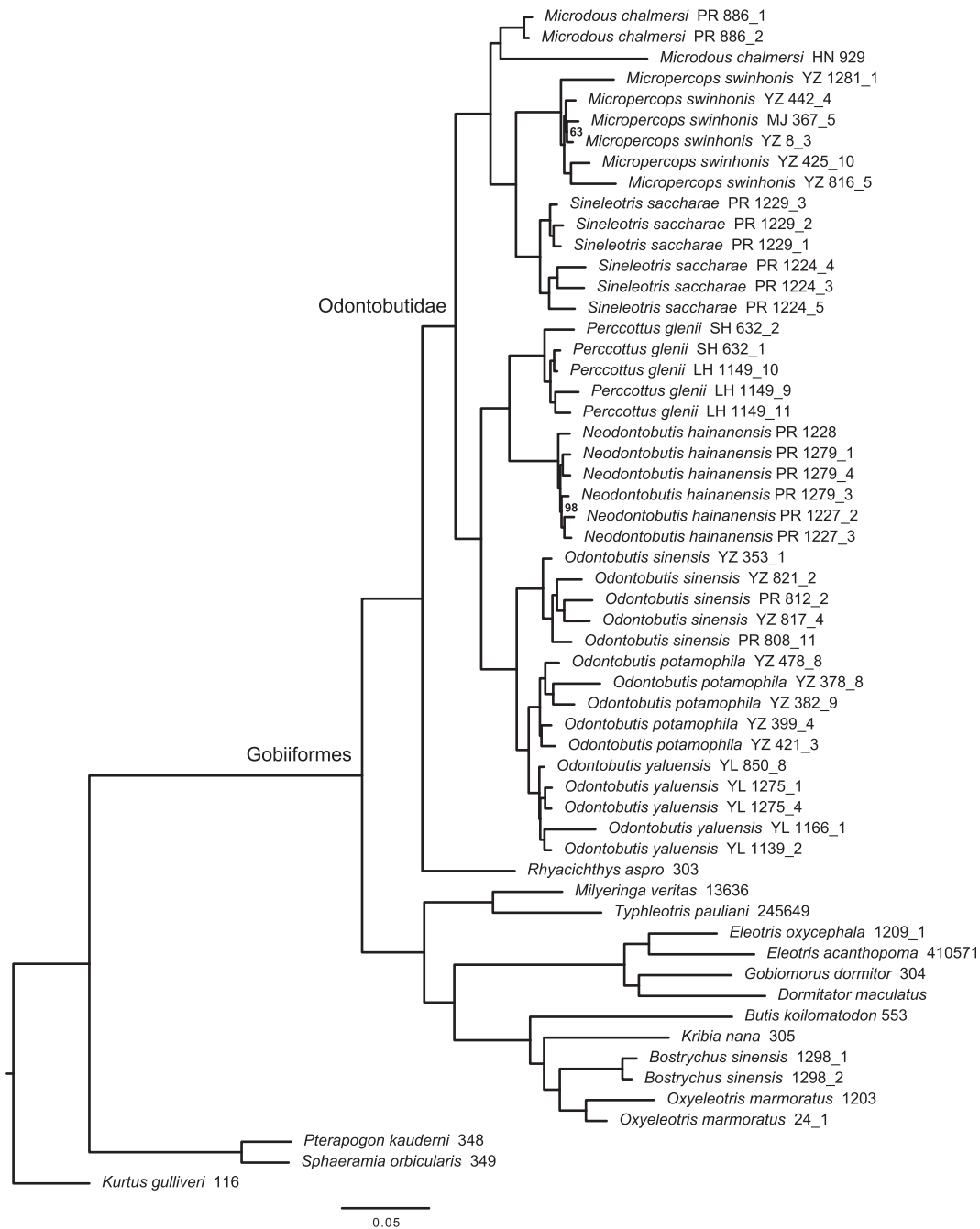
Fig. 2. Concatenated ML tree of the Odontobutidae based on the 4397 loci partitioned by the best partitioning scheme suggested by PartitionFinder. Bootstrap support values less than 100 are denoted. *Microdous*, a new genus name of *Philypnus* (synonym).
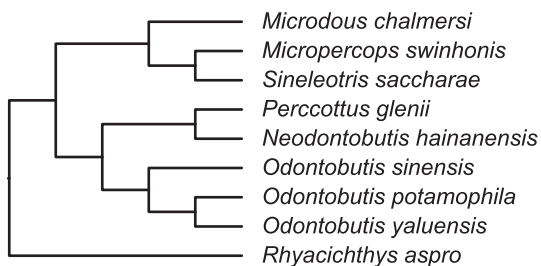

Fig. 3. A species tree obtained from ASTRAL analysis on the 4397 loci. Most outgroups (except *Rhyacichthys aspro*) not shown. All nodes are supported with 100 bootstrap values.

10.3–26.4 Ma, 95% HPDs). The divergence time between *Micropercops* and *Sineleotris* was in the late Miocene (10.6 Ma, 4.9–16.9 Ma, 95% HPDs).

3.5. Biogeographical analyses

The results of DEC analysis implemented in RASP showed that ancestor of the odontobutids originated in southern China and Indo-China Peninsula (G; GH), most possibly originated in southern China (G), and ancestor of *Odontobutis*, *Perccottus* and *Neodontobutis* occurred in southern China and northern China (G; GC). The ancestor of *Perccottus* and *Neodontobutis* was distributed in southern China (G). The ancestor of *Micropercops*, *Sineleotris* and *P. chalmersi* was distributed in southern China, and Indo-China Peninsula (G; GH). The ancestor of *Micropercops*
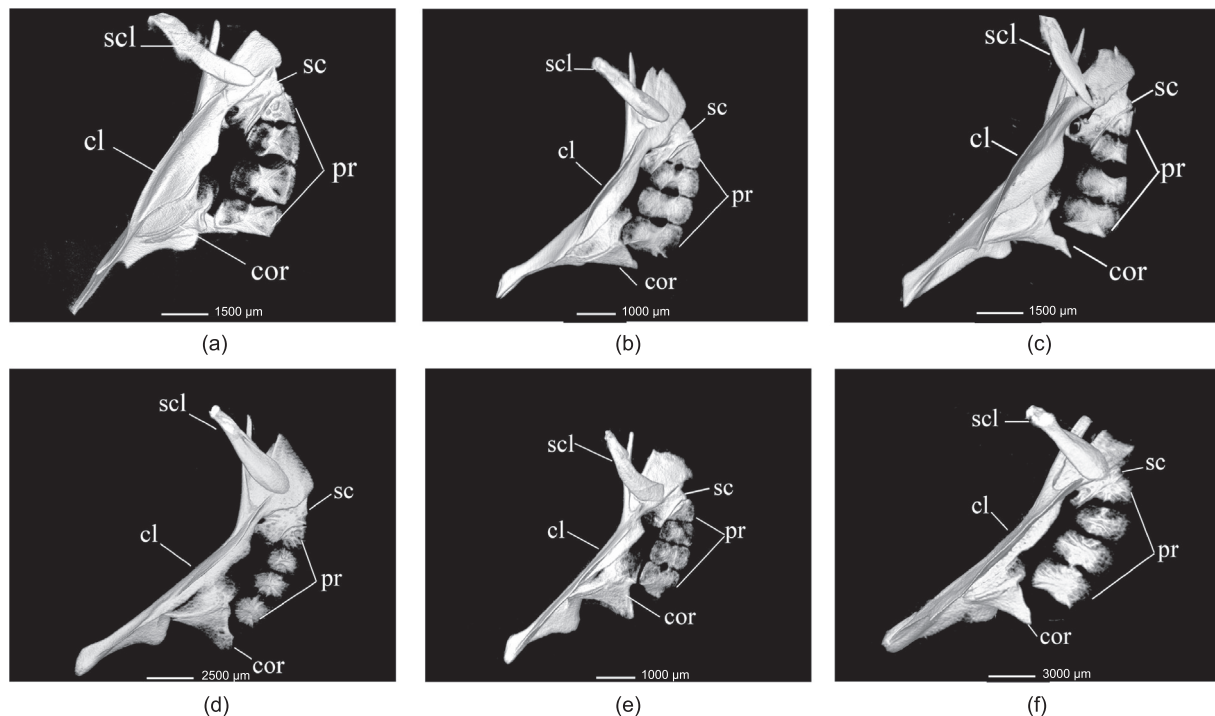
Fig. 4. Pectoral girdle of (a) *Microdous chalmersi* (CL929), (b) *Micropercops swinhonis* (CL1281_1), (c) *Sineleotris saccharae* (CL1229_3), (d) *Perccottus glenii* (CL1149_10), (e) *Neodontobutis hainanensis* (CL1279_1), (f) *Odontobutis yaluensis* (CL1139_2) in lateral view. cl: cleithrum, cor: coracoid, pr: proximal radials, sc: scapula, scl: super cleithrum.

and *Sineleotris* was found in southern China (G) (Fig. 5). The results of biogeographic analyses based on the distribution of genera are similar, except that the probability of all ancestral nodes occurring in Indo-China Peninsula increased (Fig. S2). BioGeoBEARS analyses also suggested that DEC model was the best based on AIC values, but distribution areas of many ancestral nodes were not resolved (Fig. S3).

4. Discussion

4.1. Validating the new members of the Odontobutidae

The Odontobutidae was established based on three genera (*Odontobutis*, *Micropercops* and *Perccottus*) (Hoese and Gill, 1993). Two additional genera (*Neodontobutis*, *Sineleotris*) were introduced into the family subsequently (Chen et al., 2002; Froese and Pauly, 2017; Iwata, 2011), but evidences supporting these revisions are lacking.

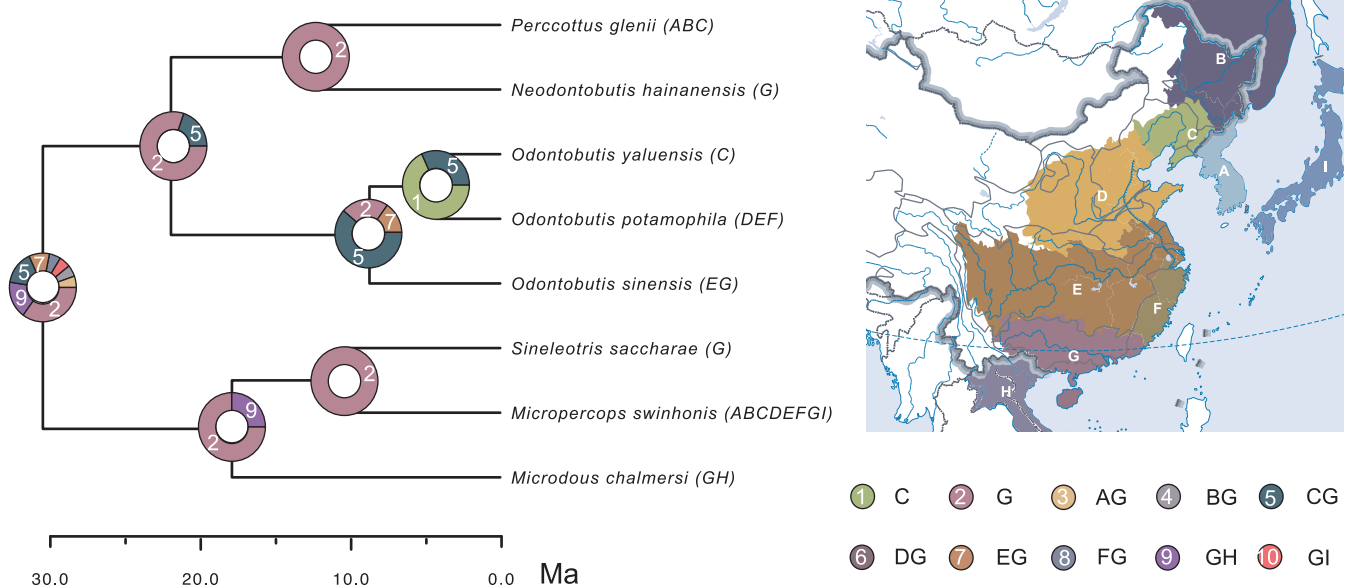Based on vomerine teeth and the absence of supraorbital ridge, *N.*

Fig. 5. Result of Lagrange Analysis (Dispersal-Extinction-Cladogenesis, DEC model) implemented in the software Reconstruct Ancestral States in Phylogenies (RASP) on the Odontobutidae. Outgroups are excluded from the analysis. Biogeographic zone: A. the Korea Peninsula; B. the Amur River, the Songhua River and Russian Far East; C. the Liaohe River and the Yalujiang River; D. the Yellow River; E. the Yangtze River and the Huaihe River; F. the Minjiang river, independent rivers in Fujian and Zhejiang provinces; G. the Pearl River and independent rivers in Hainan Island; H. the Indo-China Peninsula; I. Japan.

hainanensis was originally described as a species of *Hypseleotris*, which is a genus of the Eleotridae (Chen and Zheng, 1985). Wu and Ni (1986) renamed it as *Philypnus macrolepis* based on its vomerine teeth, protrusile lower jaw, a black stripe under ocular region. Chen et al. (2002) proposed that *Neodontobutis* shared a character of the Odontobutidae, a simple longitudinal infraorbital papillae pattern, which, however, is not a well-accepted synapomorphy of the Odontobutidae. The other more established synapomorphy of the Odontobutidae involves the condition of the pectoral girdle, in which the scapula is large, excluding proximal radial from contacting with cleithrum (Hoese and Gill, 1993; Nelson, 2006; Wu, 2008). We verified that the diagnostic pattern of the pectoral girdle of the Odontobutidae was observed in *N. hainanensis* by X-ray microtomography (Fig. 4e). Additionally, our molecular analyses provided strong evidence supporting that *Neodontobutis* belongs to the Odontobutidae (Figs. 2 and 3).

Sineleotris saccharae was originally described by Herre (1940). Chen and Zheng (1985) placed the species in genus *Philypnus* and named it *P. compressocephalus* based on vomer filled with villiform teeth, smooth gill rakers and the number of vertebrae. Wu (1991) moved the species to genus *Hypseleotris* based on toothless vomer, spineless preopercle, the absence of supraorbital ridge, the number of wide vertical bands and vertebrae, but he then changed it back to *Sineleotris* considering combined characters of cephalic sensory canals, the number of vertebrae and the width of the vertical bands (Wu, 2008). We checked two specimens of *S. saccharae* (CL1229_1, CL1224_3), and confirmed that it does not process vomerine teeth as suggested by Wu (2008). *Sineleotris* was not assigned to the Odontobutidae until Chen et al. (2002) implied it based on personal observation without any justifications. Our analyses provided the first molecular evidence confirming that *Sineleotris* is a member of the odontobutids (Figs. 2 and 3). Additionally, the microtomography indicates that *Sineleotris* does belong to the Odontobutidae (Fig. 4c).

Philypnus chalmersi and *Terateleotris* were also added to the family by Chen et al. (2002) and Nelson (2006). *Philypnus chalmersi* was described based on vomerine teeth, spineless preopercle and wide gill openings, extending to under front part of eye (Nichols and Pope, 1927). It was subsequently placed under *Perccottus* based on the presence of a spineless preopercle and presence of vomerine tooth and the number of anal fin rays (Zheng, 1981). Chen et al. (2002) was the first to point out that *P. chalmersi* is a species of *Sineleotris*, a member of the Odontobutidae but provided no evidences. Iwata (2011) checked the combination of characters of the Odontobutidae defined by Hoese and Gill (1993) and concluded that *Neodontobutis*, *Sineleotris* and *P. chalmersi* were all members of the odontobutids but the status of the species needed to be clarified. In our concatenated gene tree and species tree, the three individuals of *P. chalmersi* formed a clade sister to the clade grouping *Sineleotris* and *Micropercops* with a bootstrap support of 100 (Figs. 2 and 3). We also verified that *P. chalmersi* has the diagnostic characters of the pectoral girdle of the Odontobutidae (Fig. 4a). Based on all the evidence, we conclude that *P. chalmersi* belongs to the Odontobutidae.

Terateleotris is sometimes also classified as an odontobutid (Kottelat, 1998). Without any justification, a single specimen collected from Xe Bangfai was describe a new species of odontobutid sleeper (Kottelat, 1998), which was re-described as a new genus *Terateleotris* (Shibukawa et al., 2001). The genus was proposed not to be an odontobutids based on its lateral-line canal, preopercular canal of cephalic sensory systems connected to oculoscapular canal, and three epurals (Shibukawa et al., 2001). *Terateleotris* were not sampled in our study due to accessibility.

4.2. Taxonomic revision

Philypnus chalmersi was placed in an eleotrid genus *Philypnus* based on vomerine teeth, spineless preopercle and wide gill openings (Nichols and Pope, 1927), which was established by Cuvier and Valenciennes (1837) (Type-species: *Platycephalus dormitator* Bloch and Schneider, 1801). However, the type-species, *P. dormitator* later was found as a

junior synonym of *Gobiomorus dormitor* (Robins and Ray, 1986). In term of the conditions of vomerine teeth, preopercle and gill openings, *P. chalmersi* fits the description of *Philypnus* (junior synonym of *Gobiomorus*) (Nichols and Pope, 1927), but we have three reasons to believe that *P. chalmersi* is not a member of *Philypnus*. First, *P. chalmersi* and *G. dormitor* are far apart on our phylogenetic tree (Figs. 2 and S1). *Philypnus chalmersi* is nested within the Odontobutidae, sister to *M. swinhonis* and *S. saccharae*, whereas *G. dormitor* is grouped with other eleotrids. Second, *P. chalmersi* has a typical scapular pattern seen in the odontobutids (Fig. 4). Third, *P. chalmersi* is distributed in southern China, but *G. dormitor* is restricted to American waters.

Philypnus chalmersi was assigned to an odontobutid genus *Perccottus* (Hoese and Gill, 1993; Larson, 2001). Chen et al. (2002) moved it to the genus *Sineleotris*. Our results showed that *P. chalmersi* is sister to a clade consisting *Sineleotris* and *Micropercops* (Figs. 2 and 3). The average p-distance between *P. chalmersi* and *M. swinhonis* and between *P. chalmersi* and *S. saccharae* are 0.054 and 0.049 respectively, both are larger than that between *M. swinhonis* and *S. saccharae* (0.038), suggesting that *P. chalmersi* should be a new genus on its own.

Microdous, new genus

Type species: *Philypnus chalmersi* Nichols and Pope (1927)

Diagnostic

The genus can be distinguished from other genera of Odontobutidae by unique combination of following features: (1) presence of suborbital bone; (2) presence of complete cephalic sensory canals; (3) cuspidal and small gill rakers; (4) gill openings extending to under front part of eye; (5) absence of vertical bands on the sides; (6) absence of dark band under eye; (7) presence of an irregular black fleck on the upper part of the base of pectoral fin in preserved specimen.

Etymology

The Chinese name of *P. chalmersi* is "a sleeper with slender teeth", referring to slender and tiny teeth on the vomer of the fish, so we name the new genus as *Microdous* with etymology: Greek, micro = small + Greek, odous = teeth. Gender, m.

Microdous chalmersi (Nichols and Pope, 1927)

Synonym

Philypnus chalmersi Nichols and Pope, 1927: 390 (Locality: Nodoa, Hainan, China); Chen and Zheng, 1985: 74 (Locality: Guangdong, Guangxi and Yunnan, China).

Perccottus chalmersi Fowler, 1962: 1451 (Locality: Hainan, China); Zheng, 1981: 214 (Locality: Guangxi, China).

Material examined

CL 929 (Ichthyological Collections, Shanghai Ocean University), 1, 89.1 mm SL (standard length); Wuzhi mountain, Hainan Island, P.R. China; Shuli Song. May. 2014. HN 832,384, 1, 10.1 mm SL; HN 833,270 (Ichthyological Collections, Shanghai Ocean University), 1, 6.8 mm SL; Changjiang, Hainan Island, P.R. China; 76V9228 (Ichthyological Collections, Shanghai Ocean University), 1, 9.9 mm SL; Qiongzhong, Hainan Island, P. R. China; Hanlin Wu. May. 1976.

Diagnostic features

Anterior of head slightly depressed and posterior of body compressed. Eye large, extending outward, located in anterior half of head. Both jaws with several rows of tiny conical teeth. Gill openings extending to under front part of eye. Vertebral count 31–32. First dorsal fins VIII, second dorsal fins I/9–10 (modally 9); Anal fin I/8–9 (modally 8); pectoral fin 14–15 (modally 14). Rear tips of first dorsal fin reaching the start point of second dorsal fin. Second dorsal fin not reaching procurrent rays of caudal fin. Caudal fin and pectoral fin large, elliptical. Pelvic fins well separate and rear tip not extending anus. Longitudinal scale rows 40–46 (modally 45); transverse scale rows 14–16 (modally 14). Nape and cheek with cycloid scales. Body and opercle with few ctenoid scales. The scapula is fully ossified and well developed, which excludes the upper proximal radial from contact with the cleithrum (Fig. 4a).

Distribution

Microdous chalmersi is presently known from southern China

(Guangdong, Guangxi and Hainan island) and northern Vietnam (Table S1).

4.3. Monophyletic Odontobutidae

Based on widely separated pelvic fin buds and other characters, Iwata et al. (2001) explored the monophyly status of Odontobutidae. Many of the characters scrutinized also appear in the Eleotridae, so the monophyly of Odontobutidae could not be determined. Akihito et al. (2000) found that *M. swinhonis* and *O. obscura* were not clustered into the same clade based on mitochondrial cytochrome *b* data, thus, suggesting a non-monophyletic Odontobutidae. This unconventional result may be caused by limited data, which also was reflected in very low bootstrap support values of many nodes in their resulting tree. Many recent molecular studies support a monophyletic Odontobutidae (Agorreta et al., 2013; Chakrabarty et al., 2012; Thacker et al., 2015), but all studies only involved two or three odontobutid species and the monophyly of the family as a whole has never been really tested. Our taxon sampling includes all odontobutid genera and the newly added members except for *Terateleotris*. All of our resulting trees support a monophyletic Odontobutidae with the Rhyacichthyidae as the sister taxon. A scapula excluding proximal radial from contacting with cleithrum is a well-accepted synapomorphy of the Odontobutidae (Nelson, 2006; Wu, 2008), but the character has only being examined in *Micropercops* and *Odontobutis* in previous studies (Hoese and Gill, 1993; Shen, 2005). We investigated the pectoral girdle of *M. chalmersi, S. saccharae, P. glenii, N. hainanensis* for the first time, and found that they all have a typical pectoral girdle of odontobutids, confirming that the Odontobutidae is monophyletic (Fig. 4).

4.4. Phylogenetic relationships among the odontobutids

Studies on phylogenetic relationships among the odontobutids are scarce. The few previous studies only involved two or three genera of *Micropercops, Perccottus,* and *Odontobutis,* whereas *Sineleotris, Neodontobutis* and *Microdous* have never been under focus. *Perccottus* and the *Micropercops* were grouped together based on vertebral number and arrangement and having seven to nine first dorsal fin pterygiophores (Birdsong et al., 1988). The sister-taxon relationship between *Perccottus* and *Micropercops* was also found in mitochondrial genome analyses (Jun et al., 2016; Zang et al., 2016), but a different study using the same mitochondrial protein coding genes supported *Perccottus* and *Odontobutis* as sister group instead of with *Micropercops* (Ma et al., 2015).

We recovered two well-supported clades: a clade comprising *Microdous, Micropercops* and *Sineleotris,* and a clade grouping *Odontobutis, Perccottus* and *Neodontobutis* (Figs. 2 and 3). External and osteological traits of the odontobutids, including presence/absence of cephalic sensory canals, presence/absence of suborbital bone and pattern of gill rakers on inner part of 4th ceratobranchial are generally consistent with molecular phylogeny resulted from the present study (Iwata, 2011;Table 2). The *Perccottus-Neodontobutis-Odontobutis* clade have no or reduced cephalic sensory canals, no suborbital bone. The grouping of *Perccottus* and *Neodontobutis* is further supported by that they share the same trait in gill rakers on inner part of 4th ceratobranchial, that is they are continuous and forming one row (Table 2). A synapomorphy was also proposed about dorsal gill-arch musculature for *P. glenii, N. aurarmus* and *O. obscura* (Springer and Johnson, 2004). For the clade of *Microdous-Sineleotris-Micropercops,* no previous studies really tested their relationships, except that Chen et al. (2002) pointed out that *P. chalmersi* (= *M. chalmersi*) was a species of *Sineleotris* without any supporting data. We found out, instead, *M. chalmersi* should be a genus on its own and sister to a clade grouping *Sineleotris* and *Micropercops* (Figs. 2 and 3).

Table 2
Comparison of three characters among genera of the Odontobutidae.

Taxon	Cephalic sensory canals	Suborbital bone	Gill rakers on inner part of 4th ceratobranchial
Perccottus	Absent	Absent	Continuous and forming one row
Neodontobutis	Absent	Absent	Continuous and forming one row
Odontobutis	Absent or reduced	Absent	Separated
Sineleotris	Complete	Present	Separated
Micropercops	Moderate	Present	Separated
Microdous[a]	Complete	Present	Separated

[a] Observed in this study. The rest data were retrieved from Iwata (2011).

4.5. Divergence time and historical biogeography

There were few studies on the divergence time among the odontobutids. The divergence time between the Eleotridae and the Odontobutidae was estimated at 60 Ma (Near et al., 2013), which is similar to the results of our analyses. The divergence time between the Rhyacichthyidae and the Odontobutidae was estimated at 98 Ma (Chakrabarty et al., 2012), much older than our estimates (47.3 Ma). However, Chakrabarty et al. (2012) used mitochondrial loci, which often produce older age estimates (Dornburg et al., 2014), and the fossil they used was the problematic otolith record of the Gobiidae (Reichenbacher and Gierl, 2014). The ancestor of the Odontobutidae was estimated existing around 30.8 Ma in part of South China known as Cathaysia, including Guangxi, Guangdong, Fujian and Zhejiang, which has never been submerged since Sinian Period (Ediacaran, 541–635 Ma) (Li, 1981), probably provided favorable habitat for the diversification of the odontobutids. The change of macro-environment might induce the divergence and dispersal of the ancestor of the odontobutids in Asia since the Eocene (33.9–56.0 Ma) (e.g. the uplift of Tibetan plateau, withdrawal of Paratethys sea, global chilling in Neogene Period, thermal precipitation in East China and the development of large-scale drainage like the Yangtze River) (Zheng et al., 2014).

Our result showed that the ancestor of the odontobutids was distributed in South China (G), which might be influenced by the climate in this area. Since the Oligocene (23.03–33.9 Ma), the uplifting Nanling, massive mountains in Zhejiang and Fujian and continental plateau in Southern Yangtze area had become a barrier to harsh climate (Gu and Chen, 1987). The divergence time of *M. chalmersi, Micropercops* and *Sineleotris* was estimated at 17.9 Ma. The divergence time between *Micropercops* and *Sineleotris* was estimated at 10.6 Ma. The change of topography and climate during the Miocene (5.333–23.03 Ma) might promote sympatric speciation and species dispersal. During the period, the Himalayan-Tibetan orogen and volcanic eruptions induced the uplift of massive mountains (e.g. Nanling and Wuyi) and the formation of deeply carved valleys in South China (Jia et al., 2004; Wu et al., 2000). Simultaneously, the formation and intensification of east-tilting topography and the Asian Monsoon occurred in East Asia (Guo, 2010; Liu et al., 2001; Zheng et al., 2008). Since the Miocene (5.333–23.03 Ma), the growth of Himalayan-Tibetan orogen, global chilling and the enhanced seasonal climate had affected the distribution of fish noticeably (Wu et al., 2000; Zhang and Guo, 2005; Zhou et al., 2017). The divergence of *Odontobutis, Neodontobutis* and *Perccottus* and the divergence of *Neodontobutis* and *Perccottus* were both estimated during this period. Until then, most of the odontobutids were restricted in South China. Since the Late Miocene, the large-scale drainages such as the Yangtze River developed. The climate and topography had been affected by Himalayan-Tibetan orogen in South China. Since the Late Pliocene (2.58–3.60 Ma), the upper reaches of the Yangtze River flowed to east and connected to the middle and lower reaches of the basin (Zheng et al, 2008). During this period, the odontobutids might spread

to northern China gradually.

Since the Neogene (2.58–23.03 Ma), maritime climate had influenced these areas in the east of 110°E in China (Wan and Cao, 1992). Climate had changed in the northern region of the Yellow River, which might induce the retreatment of the warm water fishes dramatically (Li, 1981). Thus, *N. hainanensis*, *S. saccharae* and *M. chalmersi* were restricted in South China or Indo-China Peninsula. On the contrary, *M. swinhonis* and *Odontobutis* were widely dispersed in China probably due to their high adaptability. During the Quaternary glaciations, there were connections between river systems in China and Japan or Korea Peninsular (Li, 1981), so *M. swinhonis* and *Odontobutis* might spread to these regions during that period. It was interesting that *P. glenii* might have developed a mechanism of cold adaptation and survived in the North during the glacial epoch. Moreover, due to river capture between the Amur River and the Liaohe River and the connection between river systems in China and Korea Peninsular in glacial epoch (Li, 1981), *P. glenii* dispersed widely in northeastern China and nearby regions.

5. Conclusions

We confirmed that *Neodontobutis* and *Sineleotris* are members of Odontobutidae and *P. chalmersi* belongs to a new genus, *Microdous* of the Odontobutidae based on combined molecular data and morphological traits of pectoral girdle. We corroborated that the Odontobutidae is a monophyletic group and sister to the Rhyacichthyidae, comprising two clades: *M. chalmersi* sister to a group consisting of *Micropercops* and *Sineleotris*, and *Odontobutis* sister to a group unifying *Perccottus* and *Neodontobutis*. Our fossil calibration and biogeographic analyses showed that the ancestor of odontobutids was widely distributed in southern China and the diversification of the odontobutids was correlated with geographic and climatic changes in East Asia during the Oligocene and Miocene.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2017.12.026

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