



Molecular phylogenetics of the fresh water sleepers *Odontobutis* (Gobiiformes: Odontobutidae) and its implications on biogeography of freshwater ichthyofauna of East Asia

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ABSTRACT

The genus *Odontobutis* is a group of freshwater fishes endemic to East Asia. Phylogenetic relationships among the *Odontobutis* species have never been fully tested due to incomplete taxon sampling and that molecular data have not been collected in many *Odontobutis* species. In the present study, we sampled 51 specimens from all known eight *Odontobutis* species with two outgroups (*Percottus glenii* and *Neodontobutis hainanensis*). We collected sequence data of 4434 single-copy nuclear coding loci using gene capture and Illumina sequencing. A robust phylogeny of the *Odontobutis* with many individuals for each species was built, supporting the current taxonomy that all extant *Odontobutis* species are valid. The two species from Japan (*O. hikimius* + *O. obscurus*) formed an independent clade sister to the “continental odontobutids”, whereas the species from southern China (*O. sinensis* + *O. haifengensis*) separated from the rest species of the genus. Surprisingly species from the lower reaches of the Yangtze River (*O. potamophilus*) was more closely related to species from the Korean Peninsula and northeastern China than to the middle reaches of the Yangtze River, such that their relationship was ((*O. sinensis* + *O. haifengensis*)(*O. platycephala* + (*O. yaluensis* + (*O. potamophilus* + *O. interruptus*))))). Divergence time among the *Odontobutis* was estimated using 100 most clock-like loci and three fossil calibration points. The crown group of the *Odontobutis* was estimated at 9.0 Ma during the late Miocene (5.6–12.7 Ma, 95% HPDs). Ancestral range of the genus was reconstructed using Reconstruct Ancestral States in Phylogenies (RASP) and BioGeoBEARS. The result suggested that the common ancestor of modern *Odontobutis* probably was distributed in Japan, southern China or the Korean Peninsula. A series of geographical events in East Asia since the late Miocene, such as the opening of the Japan/East Sea, rapid uplift of the Tibetan Plateau and climate change in the northern region of the Yellow River might account for diversification and current distribution pattern of the *Odontobutis*.

1. Introduction

East Asia is home to about 2000 species of freshwater fishes, among which the eastern part of China, the Korean Peninsula and the Japanese Archipelago are known for their abundant freshwater fish resources (De Silva et al., 2007; Hosoya, 2022; Xing et al., 2016). Interestingly,

freshwater ichthyofauna reflect high similarity among these regions, despite their geographical isolation. Numbers of previous studies had suggested that freshwater fishes distributed in the Korean Peninsula and the Japanese Archipelago are mostly “continental origin” (Jeon et al., 2022; Yoon et al., 2018). The history of speciation, dispersal and colonization of these species in the areas, however, is under dispute, as there

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were repeated connections and separations of the freshwater system between the continent and the two regions (Kizaki, 1977; Watanabe, 2012).

The freshwater sleepers (genus *Odontobutis*), a group of typically small, benthic, ambush predators with a large head, stout body, wide rounded pectoral fins and obscure in mottled coloration can be found in small ponds, lakes and rivers (Iwata, 2011; Wu and Zhong, 2008). The freshwater sleepers are widely distributed in East Asia, so it is an ideal taxon for studying the historical biogeography and distribution patterns of freshwater ichthyofauna in East Asia. However, phylogenetic relationships within *Odontobutis* are yet resolved, which hampered us from understanding the evolutionary pattern and colonization history of this taxon.

According to Li et al. (2018), the genus *Odontobutis* are a group of “late-diverged” odontobutid fishes with the stem group estimated in early Miocene (22.3 Ma, 13.9–31.6 Ma, 95% HPDs) while the crown group were dated to late Miocene (6.6–13.7 Ma, 95% HPDs). Temminck and Schlegel (1850) described *Eleotris obscurus*, then Bleeker (1874) established the genus *Odontobutis* with *E. obscurus* as the type species, considered that a synapomorphy of this taxon was the absence of the small spines at the posterior edge of the preopercular. *Odontobutis potamophilus*, *O. haifengensis*, *O. interruptus*, *O. platycephala*, *O. yaluensis*, *O. hikimius* and *O. sinensis* were described subsequently (Chen and Zheng, 1985; Günther, 1871; Iwata et al., 1985; Iwata and Sakai, 2002; Wu et al., 2002; Wu et al., 1993). The eight species of *Odontobutis* are distributed in China, the Korean Peninsula and Japan (Table 1; Fig. 1).

The phylogeny of the genus *Odontobutis* is still controversial. Taxonomy of *Odontobutis* is mostly based on morphological evidences, while molecular analyses on the genus are scarce and the few molecular studies mainly are based on mitochondrial loci and incomplete taxa sampling. For example, molecular phylogeny based on 12S rRNA suggested that *O. potamophilus* was more closely related to *O. haifengensis* than to *O. yaluensis* (Ren and Zhang, 2007), but in another study using 12 mitochondrial protein coding genes (PCGs), *O. potamophilus* was more closely related to *O. yaluensis* (Zhong et al., 2018). Most studies agreed that *O. sinensis*, a species distributed in the Pearl River basin and the middle reach of the Yangtze River, was sister to the clade unifying the Korean species *O. platycephala*, the northeastern China species *O. yaluensis* and the eastern China species *O. potamophilus* (Hou et al., 2014; Jun et al., 2016; Mukai and Nishida, 2003; Zang et al., 2016), but a molecular phylogeny based on a set of 13 PCGs suggested that *O. sinensis* was nested with *O. platycephala* (Ma et al., 2015). Furthermore, a recent study based on same set of loci, with the additional

O. haifengensis, suggested that the *O. platycephala* was sister to a clade grouping *O. sinensis* and *O. haifengensis* (Lv et al., 2020).

Li et al. (2018) reconstructed the phylogenetic tree of the *Odontobutidae* using 4434 nuclear loci, suggesting that *O. sinensis* was sister to the clade grouping *O. yaluensis* and *O. potamophilus*. However, Li et al. (2018) only included three *Odontobutis* species in their tree, thus the phylogenetic relationship within the genus *Odontobutis* is still unclear. Complete taxon sampling, especially including the Japanese and Korean species is needed to reconstruct a robust phylogeny of the genus, and to understand their evolutionary history and distribution patterns.

In the present study, we reconstructed a phylogeny of the *Odontobutis* with a comprehensive taxon sampling including 51 individuals of all known *Odontobutis* species as well as two outgroup species. We collected sequence data from 4434 nuclear loci using targeted-gene capture and next-generation sequencing (Jiang et al., 2019; Li et al., 2013). We also calibrated the phylogeny with fossils reported from previous studies (Bannikov and Carnevale, 2016; Chakrabarty et al., 2012; Gierl and Reichenbacher, 2017; Gierl et al., 2013; Li et al., 2018). Our objectives are (1) to infer phylogenetic relationships within the genus *Odontobutis*; (2) to estimate divergence time among the *Odontobutis* species; (3) to estimate the biogeographic history of the genus.

2. Material and methods

2.1. Sampling and DNA extraction

Forty-seven individuals of all known *Odontobutis* species from China, Korean Peninsula and Japan were sampled for fin clips or muscle tissues. Each species included 5–7 individuals collected from different geographical locations. There were three to six different populations sampled for each Chinese species, covering their wide distribution areas. The Japanese and Korean species were sampled from one locality only, as their distribution areas are relatively narrow. Two *Perccottus glenii* and two *Neodontobutis hainanensis* were sampled as outgroups (Fig. 1, Supplementary Table S1). Specimens were given a voucher number (Supplementary Table S1) and deposited at the Fish Collection of the Lab of Molecular Systematic & Ecology (LMSE), Shanghai Ocean University (SOU), China. 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 using agarose gel electrophoresis.

2.2. Bait design

A suite of 4434 single-copy nuclear coding sequence (CDS) markers was adopted from previous studies (Li et al., 2018; Jiang et al., 2019). The length of target loci ranged between 120 bp and 5,161 bp. Biotinylated RNA baits of 120 bp with 2 × tiling was synthesized based on sequences of *Rhinogobius similis* (Gobiiformes) (MYcroarray, Ann Arbor, Michigan; cat#, 150901-Li-Goby). Sequences of *Periophthalmus modestus* (Gobiiformes) were used when sequences of *R. similis* were not available for some targeted loci. The sequences of targeted loci and baits are listed in Supplemental Materials (Supplementary File S2).

2.3. Library preparation, gene capture, pooling and sequencing

Library preparation and gene capture were performed 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 into 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 for each sample. Two rounds of gene capture were performed on each sample to increase on-target reads of captured loci. The samples were labeled with an 8 bp index on the P7 adapter to facilitate multiplex sequencing. Enriched and

Table 1
Distribution regions of all *Odontobutis* species.

Species	Distribution	Distribution areas implemented in biogeographic analysis
<i>Odontobutis obscurus</i> Temminck & Schlegel, 1845	Japan	A
<i>O. potamophilus</i> Günther, 1861	Eastern China: the Yangtze River, the Minjiang river and independent rivers in Fujian and Zhejiang	CD
<i>O. haifengensis</i> Chen, 1985	China: Guangdong	B
<i>O. interruptus</i> Iwata & Jeon, 1985	Korean Peninsula	F
<i>O. platycephala</i> Iwata & Jeon, 1985	Korean Peninsula	F
<i>O. yaluensis</i> Wu, Wu & Xie, 1993	China: the Liaohe River and the Yalujiang River	E
<i>O. hikimius</i> Iwata & Sakai, 2002	Japan	A
<i>O. sinensis</i> Wu, Chen & Chong, 2002	China: the Yangtze River and the Pearl River	BC

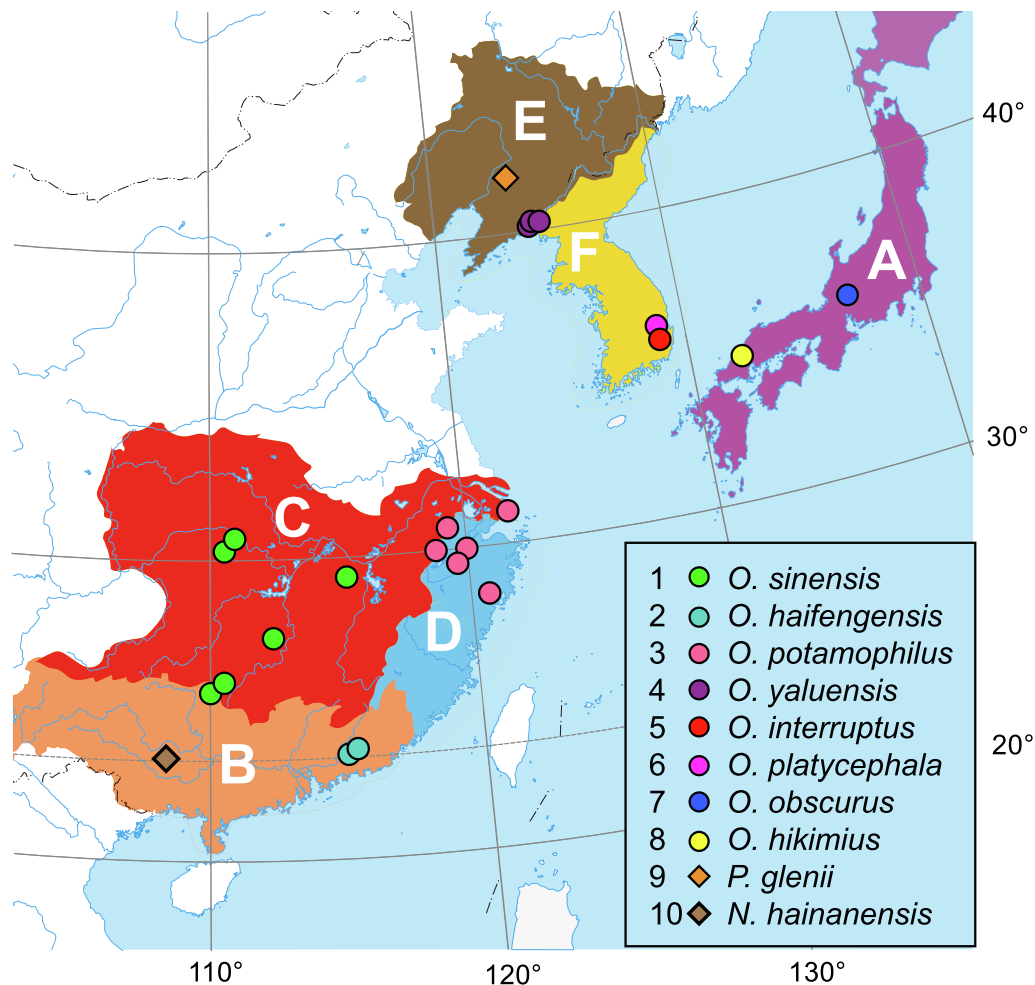


Fig. 1. Six distribution zones for biogeographic analysis with sampling localities of eight *Odontobutis* species and two outgroups. A. Japan; B. the Pearl River basin in southern China; C. the middle and lower reach of the Yangtze River basin; D. the independent rivers in Fujian and Zhejiang provinces, China; E. the Liaohe River basin and the Yalujiang River basin; F. the Korean Peninsula.

indexed products were pooled in equimolar ratios for sequencing on an Illumina HiSeq X10 lane by Annoroad (Beijing, China).

2.4. Data assembly

Reads from each sample were parsed according to their 8 bp index. Data preparation and assembly was performed according to Yuan et al. (2016). Briefly, Trim_Galore Version 0.6.7 (Krueger, 2021) and Cutadapt v1.2.1 (Martin, 2011) with default parameters were used to trim adapter sequences and low quality sites ($Q < 20$) from the reads. The resulting trimmed reads were then assembled into contigs and filtered. Locus-based output was obtained using a Perl script wrapper developed by Yuan et al., (2019), which also included a “paralog removal” protocol to eliminate potential paralogs. The putative target sequences were compared to the reference genome using BLAST, and any sequences that failed to hit the target region were considered paralogs and discarded. In the next step, loci with poor enriched data ($<50\%$) were excluded, while the remaining loci were retained for alignment. A filtered dataset consisting of 2724 loci was used for subsequent alignment. All sequences were initially translated into amino acid and aligned in batch using Clustal Omega (Sievers et al., 2011). The aligned amino acid sequences were then converted back to DNA alignment while preserving the original nucleotide information. Pairwise distance was calculated for all loci, and sequences with abnormal high distance values were discarded if they could not be corrected manually. The aligned sequences can be found in Supplemental Materials (Supplementary File S3).

2.5. Phylogenetic analyses

The aligned DNA sequences were used for reconstructing phylogeny. Maximum likelihood trees (ML tree) were reconstructed using concatenated data (477,822 bp) under GTRGAMMA model using RAxML v8.2.8 (Stamatakis, 2014) with 1000 bootstrap replicates. Data were both partitioned by codon position as well as by a partitioning scheme identified using PartitionFinder v2.1.1 (Lanfear et al., 2017). Initial data blocks of codons (Supplementary File S4) were analyzed under the GTR + G model and the rcluster algorithm in PartitionFinder v2.1.1 (Lanfear et al., 2014). The optimal partitioning scheme was determined applying BIC criterion. In addition, IQ-TREE multicore version 2.1.4-beta (Minh et al., 2020) were used to infer a ML tree for comparison, utilizing the best-fit model GTR + F + R2 as suggested by ModelFinder (Kalyaanamoorthy et al., 2017) based on the BIC value with 1000 SH-aLRT (Shimodaira-Hasegawa-like approximate likelihood ratio test) test replicates (Anisimova et al., 2011).

Each individual gene tree was reconstructed using RAxML v8.2.8 under the GTRGAMMA model (Supplementary File S5). Then, ASTRAL 5.6.3 (Mirarab et al., 2014) was used to generate a coalescent tree from all gene trees at the level of species with a full annotation parameter “-t 2” as well as an option “-q” to score the resulting coalescent tree to produce its quartet score. The “-q” option in ASTRAL is used to score an existing species tree; generate its quartet score, compute its branch lengths, and calculate its ASTRAL branch support values. “-t 2” was chosen to display quartet support and local posterior probabilities for

each branch. The resulting ML tree and coalescent tree were visualized in FigTree v.1.4.4 (Rambaut, 2018).

2.6. Estimation of divergence time

The initial data set, including eight ingroups and two outgroups and 14 related gobiiformes species from previous studies (Li et al., 2018) were assembled following the protocol described in section 2.4 to enable fossil calibration. One hundred most clock-like loci were selected from the resulting 4341 loci that had no missing data in the 24 species using a Perl script adopted from Yuan et al. (2019) (Supplementary File S6). Individual gene trees were reconstructed under both strict-clock and relaxed-clock model. The log likelihood ratio between the relaxed-clock and the strict-clock model was used to rank the loci to select the 100 most clock-like loci as described in Li et al. (2018). BEAUti v2.7.1 was used to create BEAST v2.7.1 input files written in XML (Bouckaert et al., 2019; Drummond and Rambaut, 2007). 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 used was relaxed clock log normal with the birth–death tree model. 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. 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 generations of MCMC, and parameter sampling every 1000 generations. The MCMC log files were examined in Tracer v.1.7 (Rambaut et al., 2018) to assess convergence and calculate effective sample sizes (ESS) of various parameters. Tree files from two runs were combined by LogCombiner v.2.7.1 with 20% Burn-in (Bouckaert et al., 2019). TreeAnnotator v.1.8.1 (Bouckaert et al., 2019) 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.1.4.4 was used to view the final trees.

Due to the lack of good fossil record of odontobutids (Gierl et al., 2022), three fossil gobiiforms employed in previous study (Li et al., 2018; Lv et al., 2020) were selected to establish priors based on the nodes splitting related outgroups: †*Carlomonnus quasigobius*, the oldest member of Gobiiformes with an estimated stratum age at Late Ypresian (47.8–56.0 Ma) (Bannikov and Carnevale, 2016); †*Paralates chapelcorneri*, which was similar to extant eleotrid *Gobiomorphus* dated to approximately 33.9–37.8 Ma (Gierl and Reichenbacher, 2017) and †*Lepidocottus aries* recorded at latest Oligocene epoch (23.03–27.82 Ma), which was related to the extant Butidae (Gierl et al., 2013). 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 the Gobiiformes. The hard minimum age, 47.8 Ma was based on the minimum estimated age of the oldest Gobiiform fossil, †*C. 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* was constrained with a hard minimum age, 23.03 Ma based on the minimum age estimation of †*L. aries* and a 95% soft maximum age, 37.8 Ma based on the maximum age estimate of †*P. chapelcorneri* (mean, 1.38; standard deviation, 0.8; offset, 23.03). The common ancestor of Eleotridae and Butidae 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 †*C. quasigobius* (mean, 1.78; standard deviation, 0.8; offset, 33.9). Admittedly, the latest integrative phylogenetic analyses on fossil gobioids suggested that †*C. quasigobius* should be assigned to Butidae, but they also highlighted that the positioning of †*C. quasigobius* remained speculative (Gierl et al., 2022) and hence we still treated

†*C. quasigobius* as a stem gobiiform.

2.7. Reconstruction of ancestral distribution of the genus *Odontobutis*

The ancestral state reconstruction software RASP v4.3 (Yu et al., 2020) was applied to reconstruct ancestral distribution of the genus *Odontobutis* based on three models: Statistical dispersal–vicariance analysis (S-DIVA), Lagrange (DEC) and Bayes–Lagrange (S-DEC) (Ree and Smith, 2008; Yu et al., 2015). A calibrated time tree, containing the representatives of eight *Odontobutis* species from the above BEAST analyses was used as input trees with outgroups discarded. All input trees can be found in Supplementary File S7. Six distribution areas were implemented in the analyses based on the major river systems and distribution areas of the genus *Odontobutis*: A. Japan; B. the Pearl River basin in southern China; C. the middle and lower reach of the Yangtze River; D. the independent rivers in Fujian and Zhejiang provinces, China; E. the Liaohe River basin and the Yalujiang River basin; F. the Korean Peninsula (Iwata, 2011; Wu and Zhong, 2008) (Table 1, Fig. 1). All distribution areas were considered adjacent in their historical states based on palaeogeographical and geological data (Iijima and Tada, 1990; Ogasawara, 1994; Yonekura et al., 2001). The distribution state of all *Odontobutis* species was loaded via the option “Load States (Distribution)”. Three models were tested on trees independently. The number of maximum distributions was constrained as three. Additionally, R package BioGeoBEARS with six models implemented in RASP (Supplementary Table S2): DEC, DEC + J, DIVALIKE, DIVALIKE + J, BAYAREALIKE and BAYAREALIKE + J with the same numbers of maximum distribution range constrained was employed to infer probabilistic historical biogeography (Matzke, 2013; Matzke, 2018) as a comparison to the results from RASP analyses. The models were ranked by AIC scores, and result from the best model was presented.

3. Result

3.1. Phylogenetic relationship

The RAxML tree based on the best partitioning scheme identified by PartitionFinder was consistent with the result using codon positions as the partitioning scheme (Fig. 2, Supplementary File S8). Bootstrap values of the ML tree were 100% for most branches, except the branch grouping *O. sinensis* and *O. haifengensis* (88%) and the branch grouping *O. yaluensis*, *O. potamophilus*, *O. interruptus*, *O. platycephala*, *O. sinensis* and *O. haifengensis* (73%). The ML tree inferred using IQ-TREE was consistent with RAxML resulting trees except for some difference in node support values (Fig. 2, Supplementary File S9). The the SH-aLRT test value of the branch including *O. sinensis* and *O. haifengensis* was 53.7%, while the branch including *O. yaluensis*, *O. potamophilus*, *O. interruptus*, *O. platycephala*, *O. sinensis* and *O. haifengensis* was 97.1%. The coalescent tree reconstructed using ASTRAL also was consistent with the ML trees (Fig. 3, Supplementary File S10). The support values of the coalescent tree were 1 (or 100%) for most branches, except for the branch splitting (*O. yaluensis*, *O. potamophilus*, *O. interruptus*, *O. platycephala*) and (*O. sinensis* and *O. haifengensis*), which was 0.7 (or 70%). The normalized quartet score of ASTRAL analysis was 0.45.

In the resulting phylogeny (Figs. 2 and 3), all *Odontobutis* species were monophyletic. The clade grouping the two Japanese species *O. hikimius* and *O. obscurus* was sister to other “continental” odontobutids. The clade of *O. sinensis* and *O. haifengensis* was sister to the clade unifying *O. platycephala*, *O. yaluensis*, *O. potamophilus* and *O. interruptus*. *Odontobutis platycephala* was sister to the clade in which *O. yaluensis* was close related to the sister group of *O. potamophilus* and *O. interruptus*. The phylogenetic relationship of the genus *Odontobutis* was (*O. hikimius* + *O. obscurus*) + ((*O. sinensis* + *O. haifengensis*) + (*O. platycephala* + (*O. yaluensis* + (*O. potamophilus* + *O. interruptus*)))).

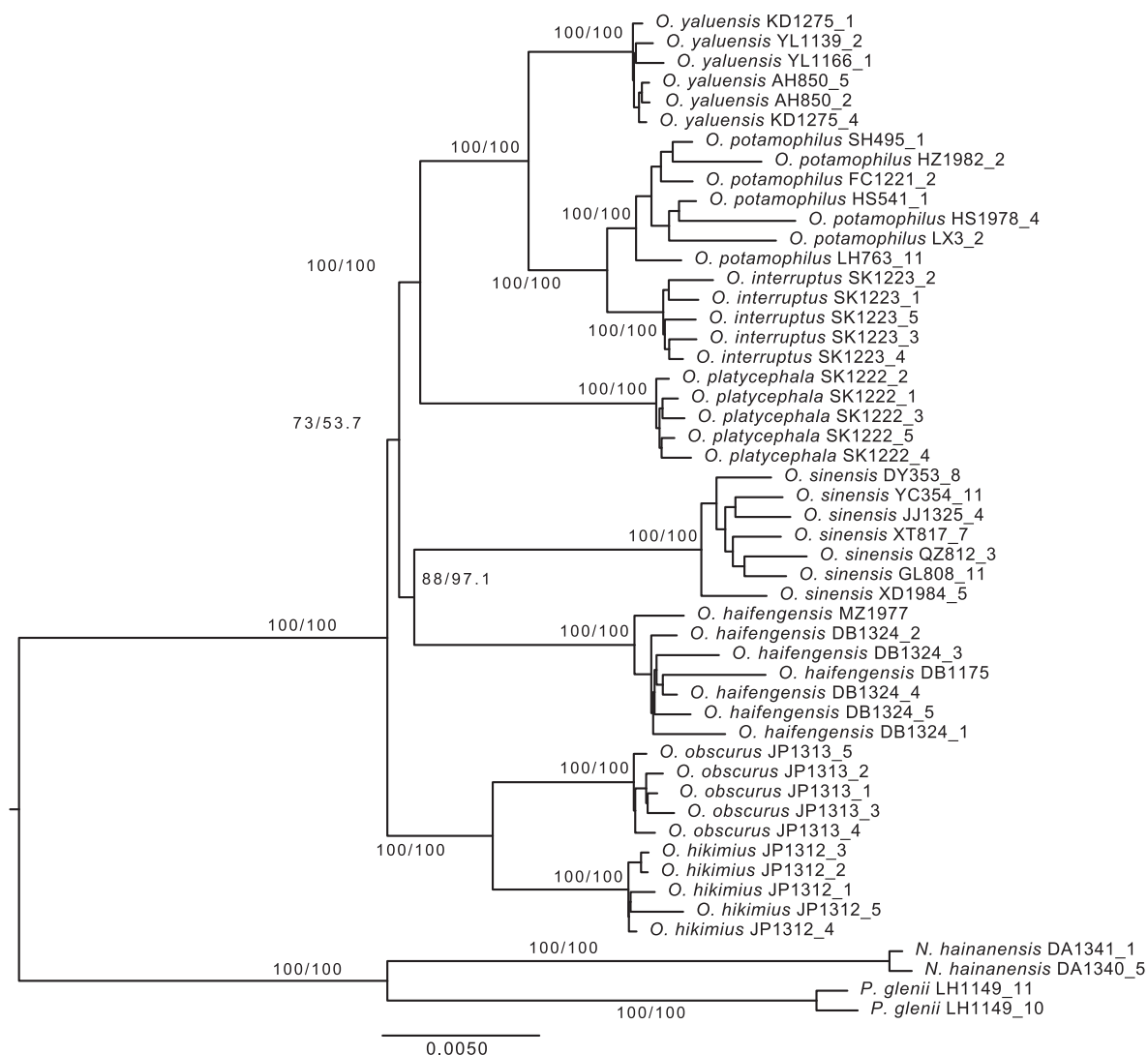


Fig. 2. Maximum likelihood tree of *Odontobutis* based on 2724 nuclear loci (477,822 bp), partitioned by the best partitioning scheme suggested by PartitionFinder. Numbers at the nodes show bootstrap supports from RAxML analysis and SH-aLRT test values from IQ-TREE (%).

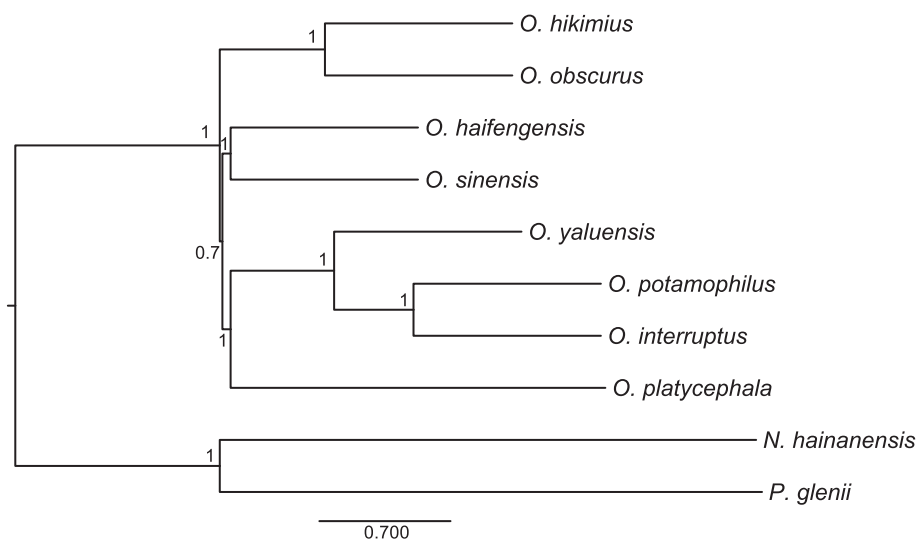


Fig. 3. A coalescent tree of the *Odontobutis* resulted from ASTRAL analysis on 2724 nuclear loci. Numbers at nodes show the branch support values.

3.2. Divergence time estimates

Both independent BEAST runs reached stationarity. ESS values of all parameters were greater than 450. The mean divergence times and their 95% higher posterior densities (HPDs) intervals are shown in Fig. 4. The ancestor of the crown group of *Odontobutis* was estimated at 9.0 Ma during the late Miocene (5.6–12.7 Ma, 95% HPDs). The divergence time between *O. hikimius* and *O. obscurus* was inferred within the Pliocene (4.5 Ma, 1.8–7.7 Ma, 95% HPDs). The common ancestor of continental *Odontobutis* was dated to the late Miocene (8.0 Ma, 4.9–11.4 Ma, 95% HPDs). The divergence time between *O. sinensis* and *O. haifengensis* was estimated at 7.1 Ma during the late Miocene (4.1–10.5 Ma, 95% HPDs). *Odontobutis platycephala* was also dated to the late Miocene (7.3 Ma, 4.3–10.5 Ma, 95% HPDs). *O. yaluensis* diverged from *O. potamophilus* and *O. interruptus* around 3.8 Ma. (1.9–5.9 Ma, 95% HPDs). The divergence time between *O. potamophilus* and *O. interruptus* was inferred as in the Pleistocene, Quaternary Epoch (1.6 Ma, 0.6–2.8 Ma, 95% HPDs).

3.3. Historical biogeography of *Odontobutis*

The results of S-DIVA analysis implemented in RASP were largely consistent with that generated from DIVALIKE model with the lowest AIC value using BioGeoBEARS (Supplementary File S11, Fig. 5). The distribution of common ancestor of *O. yaluensis*, *O. potamophilus* and *O. interruptus* had equal probabilities in three scenarios: the independent rivers in Fujian and Zhejiang provinces, the Liaohe River basin and the

Yalujiang River basin and Korean Peninsula (DEF); the middle and lower reach of the Yangtze River basin, the Liaohe River basin and the Yalujiang River basin and Korean Peninsula (CEF); the Liaohe River basin and the Yalujiang River basin and Korean Peninsula. Similarly, the ancestral distribution of the common ancestor of *O. potamophilus* and *O. interruptus* also had three distribution ranges with same probability values: CF, DF, CDF. The results of DEC and S-DEC analysis were both consistent with the S-DIVA analysis but with lower probability values on some nodes (Supplementary File S12 and S13). The BioGeoBEARS result suggested a scenario that the distribution of the common ancestor of *Odontobutis* was estimated with equal probability in Japan, the Pearl River basin and the Korean Peninsula (ABF). The common ancestor of *O. hikimius* and *O. obscurus* was distributed in Japan (A), whereas the common ancestor of others *Odontobutis* was distributed either in southern China and the Korean Peninsula (BF). The ancestor of *O. sinensis* and *O. haifengensis* occurred in the Pearl River basin in southern China (B). The ancestor of *O. yaluensis*, *O. potamophilus*, *O. interruptus* and *O. platycephala* was found in the Korean Peninsula (F). The ancestor of *O. potamophilus*, *O. interruptus* and *O. platycephala* was found in the Liaohe River and the Yalujiang River basin and the Korean Peninsula. The ancestor of *O. potamophilus* and *O. interruptus* was distributed in the middle and lower reaches of the Yangtze River basin, the independent rivers in Fujian and Zhejiang provinces and Korean Peninsula (CDF) (Fig. 5).

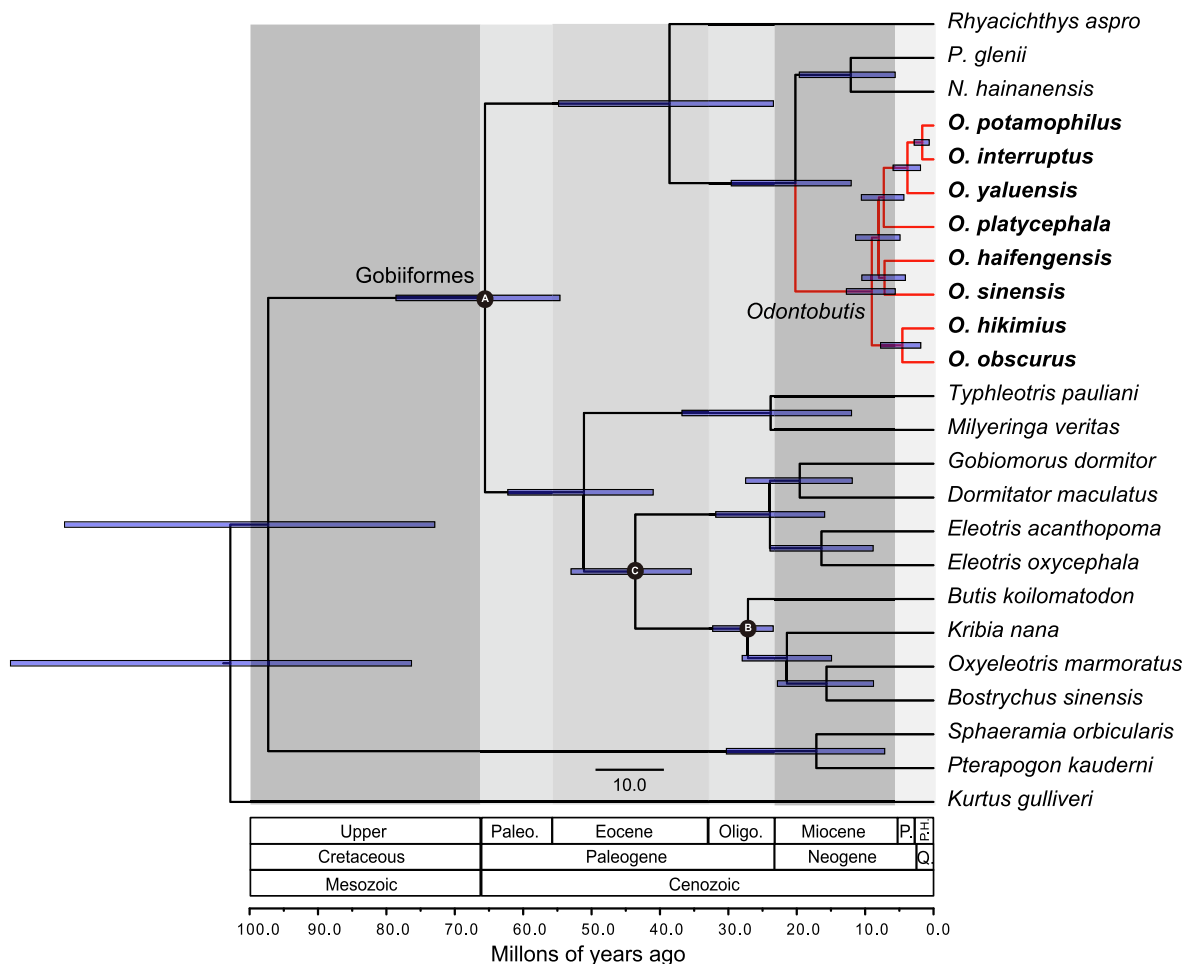


Fig. 4. Divergence time estimation of the *Odontobutis* from BEAST analysis based on 100 most clock-like loci. Fourteen closely related outgroups were included for fossil calibration. Shaded bars on the nodes represent 95% highest posterior credible age intervals for each node. Black dots with letters (A, B and C) represent fossil calibration points (A: †*Carlomonnus quasigobius*, B: †*Paralates chapelcorneri*, C: †*Lepidocottus aries*).

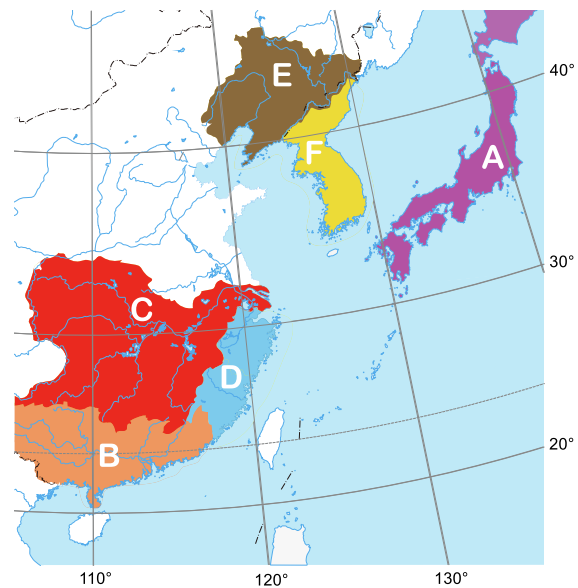
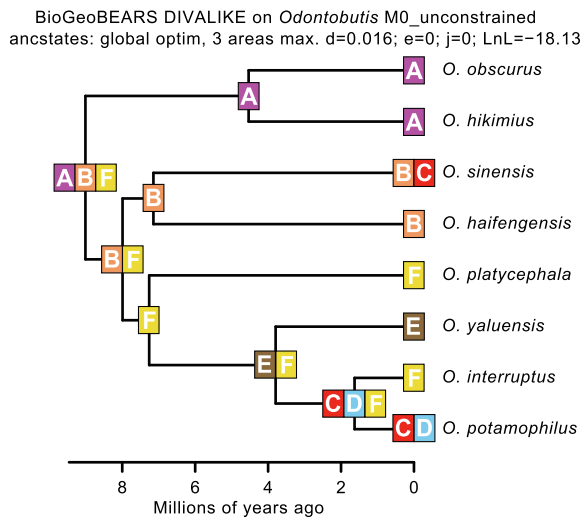


Fig. 5. Calibrated time tree of the *Odontobutis* with estimated distribution range of the nodes under DIVALIKE model implemented in BioGeoBEARS. Outgroups were excluded from analysis. Biogeographic zones: A. Japan; B. the Pearl River basin; C. the middle and lower reach of the Yangtze River basin; D. the independent rivers in Fujian and Zhejiang provinces of China; E. the Liaohe River basin and the Yalujiang River basin; F. the Korean Peninsula.

4. Discussion

4.1. Phylogenetic relationships among the *Odontobutis* species

The resulting phylogeny suggested that all *Odontobutis* species are monophyletic with 100% of bootstrap support. However, the support value for the branch defining the most recent common ancestor of *O. yaluensis*, *O. potamophilus*, *O. interruptus*, *O. platycephala*, *O. sinensis* and *O. haifengensis* was relatively low (Figs. 2 and 3). This, together with the short branch length and the low normalized quartet score (0.45) of ASTRAL analyses suggested high-level of incomplete lineage sorting (ILS) in the clade, or it could be resulted from ancient introgression events among those taxa. Currently, the direction and inherited proportion of the gene flow(s) is unclear, calling for further work focusing on reticulate evolutionary history of the *Odontobutis*.

Previous studies on phylogenetic relationships among the *Odontobutis* species were mostly based on mitochondrial sequences and only involved a few species (Hou et al., 2014; Jun et al., 2016; Lv et al., 2020; Ren and Zhang, 2007; Zang et al., 2016; Zhong et al., 2018). Thus, the phylogenetic relationships of this genus are largely unresolved and inconsistent among different studies. Ki et al. (2008) suggested a phylogenetic relationship of (*O. obscurus* + (*O. interruptus* + *O. platycephala*)) in their resulting Bayesian inference (BI) tree, while Niu et al. (2017) suggested the opposite scenario that was (*O. platycephala* + (*O. yaluensis* + (*O. interruptus* + (*O. obscurus* + *O. potamophilus*))))). Our results are generally in agreement with that of Ki et al. (2008) except that the placement of *O. obscurus* is consistent with that of Niu et al. (2017). Such phylogenetic relationship is also supported by some morphological evidence, such as the cephalic sensory canal pores C at postocular are absent in *O. obscurus*, but present in *O. platycephala*, *O. yaluensis*, *O. interruptus* and *O. potamophilus* (Iwata et al., 1985). Ma et al. (2015) suggested that *O. sinensis* is sharing a most recent common ancestor with *O. platycephala*, forming a clade sister to the clade grouping *O. yaluensis*, *O. interruptus* and *O. potamophilus*. In our resulting phylogeny, *O. sinensis* and *O. platycephala* are in two distinct clades: *O. sinensis* was grouped with *O. haifengensis* while *O. platycephala* shared a most recent common ancestor with the clade grouping *O. yaluensis*, *O. interruptus* and *O. potamophilus*. Ren and Zhang (2007) suggested that *O. sinensis* was sister to all other *Odontobutis* species, which were split into two clades: ((*O. yaluensis* + (*O. obscurus* +

O. hikimius)) + (*O. potamophilus* + *O. haifengensis*)). However, their results were based on a partial sequence of mitochondrial genes (12S rRNA) with low support for the two clades, 49% and 48% respectively. Our analyses supported the close relationship between *O. obscurus* and *O. hikimius*, but *O. sinensis* was sister to *O. haifengensis*. *Odontobutis yaluensis* was closely related to *O. potamophilus* instead of *O. obscurus* or *O. hikimius*, which are also supported by a morphological evidence that the sensory papillae pattern at the end of the sensory pitlines L5 in *O. obscurus* and *O. hikimius* are arranged in a line but are scattered in patches in *O. yaluensis* (Iwata, 2011; Wu and Zhong, 2008). The placement and divergence of *O. haifengensis* also was controversial among previous studies. Ren and Zhang (2007) suggested that *O. haifengensis* and *O. potamophilus* were sister species but Zhong et al. (2018) suggested that *O. haifengensis* diverged directly from the most recent common ancestor of *O. sinensis*, *O. platycephala*, *O. yaluensis*, *O. interruptus* and *O. potamophilus*. Latest studies (Lv et al., 2020) suggested a different scenario that *O. haifengensis* were grouped with *O. sinensis*, whereas *O. platycephala* was a sister to a clade containing *O. haifengensis* and *O. sinensis* and it then sister to a clade containing *O. yaluensis*, *O. interruptus* and *O. potamophilus*. Our results indicated that *O. haifengensis* and *O. sinensis* were grouped together and split earlier from all other species, and then a clade of the two Japanese species branched off. For the rest species, *O. platycephala* was sister to a clade containing *O. yaluensis*, *O. interruptus* and *O. potamophilus*. Although the results of Lv et al. (2020) were supported with high bootstrap value, their trees were based on 13 mitochondrial PCGs, which essentially is a single linked locus. In contrast, our analysis was based on 4434 independent nuclear loci and it is consistent with the “south-to-north” divergence trend of the odontobutids suggested by Li et al. (2018).

4.2. Taxonomic implications

The genus *Odontobutis* was established by Bleeker (1874) with *Odontobutis obscurus* (*Eleotris obscura*) as the type species. *Odontobutis potamophilus*, *O. haifengensis*, *O. interruptus*, *O. platycephala*, *O. yaluensis*, *O. hikimius* and *O. sinensis* were added to this genus subsequently based on morphological characters. Molecular evidences supporting these revisions were few, such as mitochondrial sequence data with scattered taxon sampling (3–5 species) (Hou et al., 2014; Jun et al., 2016; Lv et al., 2020; Ma et al., 2015; Ren and Zhang, 2007; Zang et al., 2016; Zhong

et al., 2018).

Due to high similarity in morphological characters, the taxonomic relationship of the genus is still controversial. *Odontobutis sinensis* had been regarded as *O. obscurus* in previous studies until Wu et al. (2002) described it as a new species according to morphological evidence. Wu et al. (2002) proposed a common character of *O. sinensis* and *O. obscurus*, the absence of cephalic sensory canal pores at postocular, including other differences, such as the number of dorsal spines and number of transverse scales. Our results showed that the five *O. obscurus* individuals from Japan (Supplementary Table S1, Fig. 2) formed a monophyletic clade with bootstrap support of 100, and the seven *O. sinensis* individuals from the Pearl River basin and the middle reach of the Yangtze river basin (Supplementary Table S1) were grouped together with a bootstrap support of 100 as well (Fig. 2). Our results are consistent with the current taxonomy that *O. sinensis* is a valid species and should be different from *O. obscurus* (Wu et al., 2002). Similarly, *O. interruptus* used to be regarded as a subspecies of *O. obscurus* (Iwata et al., 1985), but both our results and results from Sakai et al. (1993) suggested that they are two distinct species.

Günther (1871) distinguished *O. potamophilus* from *O. obscurus* by two black flecks on the upper part of the base of pectoral fin and regarded it as a new species of *Odontobutis*. In fact, however, two black flecks on the upper part of the base of pectoral fin are absent in some *O. potamophilus* specimens (Wu and Zhong, 2008), so this character cannot be used to separate *O. potamophilus* from *O. obscurus*. Iwata et al. (1985) proposed that *O. potamophilus* possess a cephalic sensory canal pores at postocular as well as that its cephalic sensory pitlines L6 and L7 were not connected, but Wu et al. (2002) argued that the cephalic sensory pitlines L6 and L7 of *O. potamophilus* were connected by inspecting a larger set of specimens. Previous studies based on mitochondrial sequence (Ren and Zhang, 2007) as well as our molecular analyses firmly supported the split between *O. obscurus* and *O. potamophilus*.

Other *Odontobutis* species (*O. haifengensis*, *O. platycephala*, *O. yaluensis* and *O. hikimius*) were all described with their unique characters despite highly morphological similarity. Additionally, our molecular analyses, together with the complete taxon sampling from different locality (Fig. 1), provided strong evidence supporting the current taxonomy of *Odontobutis*, that extant *Odontobutis* includes eight distinct species.

4.3. Divergence time and historical biogeography

Sakai et al. (1993) estimated Nei's genetic distance (D) among three *Odontobutis* species (*O. obscurus*, *O. platycephala* and *O. interruptus*), between 1.053 and 1.230, suggesting that the divergence between these three species was about 5–6 million years ago. Ren and Zhang (2007) also proposed that the divergence of the genus *Odontobutis* was in the middle Pliocene (4.9–6.5 Ma) based on their resulting p-distance (0.098–0.133) among six *Odontobutis* species. Both the above mentioned analyses did not involve fossil calibration. In the latest phylogenetic analysis, Lv et al. (2020) inferred that the crown group of *Odontobutis* was in the late Oligocene (24.9 Ma, 95%, CI: 21.3–28.5 Ma), which is much earlier than the late Miocene (6.6–13.7 Ma, 95% HPDs) estimated by Li et al. (2018) and the late Miocene (5.6–12.7 Ma, 95% HPDs) found in present study (Fig. 4). These differences might be due to the result from Lv et al. (2020) was based on mitochondrial genes, which usually generate older age estimates (Dornburg et al., 2014).

Sakai et al. (1998) hypothesized that there once existed a massive freshwater system (Second Setouchi Basin) connecting the freshwater system of eastern China with the Japanese Archipelago, based on the conclusion that the *O. obscurus* were found in both eastern China and Japan but not in the Korean Peninsula, where the ancestor of *O. obscurus* and other *Odontobutis* species already having diverged. The ancestral populations of *O. obscurus* in two places separated due to the separation of the East China Sea from the massive freshwater system that started in

the middle Pliocene (about 5 Ma). However, with the description of the Chinese endemic species *O. sinensis*, the hypothesis was not supported (Ren and Zhang, 2007). Ren and Zhang (2007) proposed another hypothesis based on their analysis that the most recent common ancestor of *Odontobutis* were widely distributed in eastern China, Korean Peninsula and Japanese Archipelago, and they diverged as the separation of the East China Sea from the continental freshwater system during middle Pliocene (4.9–6.5 Ma). Li et al. (2018) estimated the ancestor distribution range of three *Odontobutis* species included the Liaohe River, the Yalujiang River and the Pearl River. Our estimated distribution of the common ancestor of *Odontobutis* included Japan (A), the Pearl River basin in southern China (B) and Korean Peninsula (F) (Fig. 5), which is roughly in agreement with Ren and Zhang (2007). Additionally, our biogeographic analyses results were different from Li et al. (2018) that the distribution range of the common ancestor of *O. yaluensis* and *O. potamophilus* expanded from the Liaohe River and the Yalujiang River to the Liaohe River, the Yalujiang River and the Korean Peninsula. Instead, the distribution range of the common ancestor of *O. yaluensis*, *O. potamophilus* and *O. sinensis* probably was in the Korean Peninsula or the Pearl River basin (Fig. 5). Such inconsistent results might be due to the additional samples of the Japanese and Korean *Odontobutis* species which were absent in the previous studies.

We have to acknowledge that either Li et al. (2018) or the present study implemented models that assumed no extinction events and equal probability of transition between regions while ignoring the distance effect, which might influence dispersal. These limitations might have a great impact on the reconstructed historical biogeography. However, since the historic distance and adjacency between the biogeographic areas remain unclear, it is necessary to take consideration of all possible dispersal among regions. Similarly, the extinction events of *Odontobutis* are uncertain, as there are no well-preserved fossil records. Additionally, our biogeographic analyses did not consider the influence of artificial canals, which may break down natural biogeographic barriers among two basins (Lynch et al., 2011; Qin et al., 2019). Some *Odontobutis* species could disperse via those connective canals and colonize another region. The Ling Canal, constructed in 214 BCE, connected the Pearl River basin with the middle reach of the Yangtze River basin, where both *O. sinensis* are found, but there is no exact evidence supporting that the current distribution of *O. sinensis* was facilitated by the Ling Canal. Nevertheless, compared with the estimated time scale of species divergence, the existing time of artificial canals is too short to make any contribution in the current distribution patterns of *Odontobutis* species.

According to Li et al. (2018), the ancestor of the *Odontobutidae* was originated from southern China around 30 Ma and dispersed northward subsequently. The growth of Himalayan-Tibetan orogen, global chilling and enhanced seasonal climate change had affected the distribution of fish noticeably since the Miocene (5.333–23.03 Ma) (Wu et al., 2000; Zhang and Guo, 2005; Zhou et al., 2017), when the stem group *Odontobutis* species dispersed widely in East Asia during this period. Palaeogeographical and geological data suggested that the Japanese Archipelago had been adjacent to the Eurasia till the middle Miocene (19–17 Ma) (Iijima and Tada, 1990; Ogasawara, 1994; Yonekura et al., 2001), which provided a path for the eastward spread of freshwater fish. In addition, a detailed examination of Neogene fossil of freshwater fishes in Japan suggested that the modern Japanese fish faunas should be considered a consequence of the successive evolutionary development of the faunas since the beginning of the Japanese Archipelago, between the Early to Middle Miocene (Nakajima, 1986; Watanabe, 2012). Our biogeographic analysis suggested that the common ancestor of *Odontobutis* were widely distributed in Japan, southern China or the Korean Peninsula (Fig. 5). By the middle Miocene (16–14 Ma), the vanishing of the freshwater system between Japan and China mainland as well as the opening of Japan Sea (Chinzei, 1986) blocked the connection of the freshwater species, including the freshwater-restricted *Odontobutis*. Accordingly, the species on the Japanese Archipelago evolved independently into the common ancestor of *O. obscurus* and *O. hikimius*.

During the late Miocene and early Pliocene, the topographical structure of the Japanese Archipelago greatly altered due to the volcanic activity, transgression and geomagnetic activities, around the Plio–Pleistocene, when the uplift and subsidence of lands were especially active (Tanaka and Nozawa, 1977; Watanabe, 2012; Yonekura et al., 2001). The divergence between *O. obscurus* and *O. hikimius* occurred under this condition, with the time scale consistent with our divergence time estimation (Fig. 5).

The continental *Odontobutis* diverged during the middle and late Miocene. The *Odontobutis* species distributed in southern China dispersed and diverged as the geomorphological and environmental changes occurred since the late Miocene, such as the development of the Yangtze River, the climate and topography change affected by Himalayan–Tibetan orogeny in Southern China (Jia et al., 2004; Wu et al., 2000). The wide-spread species *O. sinensis* might disperse northward or westward to the Yangtze River basin during this period. Since the late Pliocene (2.58–3.60 Ma), the elevation change caused by the rapid uplift of the Tibetan Plateau had led to changes in hydrological environment (such as flow velocity) in the upper reach of the Yangtze River (Li and Pan, 2002), forming biogeographic barriers that block the dispersal of fishes with weak swimming ability, such as *O. sinensis*, to the upper reach of the Yangtze River basin. Meanwhile, as the upper reaches of the Yangtze River flowed eastward and connected to the middle and lower reaches of the basin (Zheng et al., 2008), thus the distribution range of *O. sinensis* expanded to the middle reach of the Yangtze River basin accordingly. *Odontobutis haifengensis*, in contrast, were only found in the subtropical region in Guangdong, which might be due to its lower tolerance to low-temperature and the blocking effect of the Nanling Mountains. Simultaneously, the *Odontobutis* species distributed in the Korean Peninsula diverged into a new species *O. platycephala* while the common ancestor of *O. yaluensis*, *O. potamophilus* and *O. interruptus* dispersed westward to the northeastern part of China via the freshwater system between eastern China and Korean Peninsula. Since the Neogene (2.58–23.03 Ma), the maritime climate 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 basin, which might induce retreatment of warm water fishes dramatically (Li, 1981). The cold-adapted species *O. yaluensis* might diverge under this condition and be restricted to the Yalujiang River basin and nearby region due to the climate. The common ancestor of *O. potamophilus* and *O. interruptus* in the Korean Peninsula was dated to the same period. The ancestor of *O. potamophilus* colonized the lower reach of the Yangtze River and independent rivers in Fujian and Zhejiang provinces from the Korean Peninsula via the connection between river systems in China and the Korean Peninsula in Quaternary glacial epoch, while the Korean species *O. interruptus* stayed in the Korean Peninsula. The high similarity of their morphology (Iwata et al., 1985) with the difference of geographical distribution strongly confirmed that *O. potamophilus* and *O. interruptus* were diverged from a common ancestor. Interestingly, despite the difference in their divergence time, the current distribution patterns of the two Korean *Odontobutis* species (*O. platycephala* and *O. interruptus*) overlap (Ren and Zhang, 2007). We hypothesized that the two Korean species might disperse and contact secondarily via the historical flood or river capture events in the Korean Peninsula, an investigation in population history of these two species would be helpful in testing this hypothesis.

5. Conclusions

Our phylogenetic tree, reconstructed using multi-locus nuclear genes from all extant *Odontobutis* species, supported that all currently described species of *Odontobutis* are distinct and valid. The phylogeny of the genus is as follows: (*O. hikimius* + *O. obscurus*) + ((*O. sinensis* + *O. haifengensis*) + (*O. platycephala* + (*O. yaluensis* + (*O. potamophilus* + *O. interruptus*))))). Fossil calibration and biogeographic analyses revealed that the ancestor of *Odontobutis* was widely distributed in Japan,

southern China or the Korean Peninsula. Species of the genus *Odontobutis* subsequently dispersed and diverged in accordance with a series of geographical events in East Asia since the late Miocene, including the opening of the Japan Sea, the rapid uplift of the Tibetan Plateau and the climate change in the northern regions of Eastern China.

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CRediT authorship contribution statement

Jiantao Hu: Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Hongjie Li:** Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Harumi Sakai:** Resources, Writing – review & editing. **Takahiko Mukai:** Resources, Writing – review & editing. **Ho Young Suk:** Resources, Writing – review & editing. **Chenhong Li:** Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2023.107871>.

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