



Molecular systematics of *Pampus* (Perciformes: Stromateidae) based on thousands of nuclear loci using target-gene enrichment

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ARTICLE INFO

Keywords:

Pampus
Silver pomfret
Taxonomy
Phylogeny
Osteological structure
Micro-CT

ABSTRACT

Pomfret fishes of the genus *Pampus* are commercially important in the Indo-Pacific region, yet the phylogenetic relationships and taxonomy of *Pampus* remain contentious. Here, we sampled 151 specimens, representing all known species of the genus, as well as two outgroup species (two families). We collected sequences from 17,292 single-copy nuclear coding loci using target-gene enrichment and Illumina sequencing for a subset samples of *P. echinogaster*, *P. argenteus*, *P. cinereus*, *P. liuorum*, *P. chinensis*, *P. minor*, and *P. punctatissimus*, which were carefully examined according to their species descriptions. Concatenated gene tree and species tree analyses resulted in identical and highly supported phylogenies, in which *P. argenteus* was sister to *P. minor* in one clade and *P. cinereus* sister to *P. chinensis* and *P. punctatissimus* in the other clade. Phylogenetic reconstruction using sequences of cytochrome c oxidase subunit I (COI) collected by us and those retrieved from NCBI suggests extensive misidentification of *Pampus* species in the NCBI database. We also measured morphological characters of each species as well as observed their osteological structure using micro-CT. Both molecular and morphological results suggest that *P. echinogaster* is a synonym of *P. argenteus*, and *P. liuorum* is a synonym of *P. cinereus*. *Pampus cinereus* from China, Bangladesh and an uncertain origin were grouped into three clades according to their sampling localities, but we could not find decisive morphological characters to describe the “cryptic species” of *P. cinereus*. Finally, based on the results of the molecular analyses and morphological reexamination, we created an identification key for the genus of *Pampus*.

1. Introduction

Pomfret fishes (genus *Pampus*) are widely distributed along the coasts of the Northwest Pacific and north Indian Oceans where they are exploited by regional fisheries. In China, 345,728 tons were harvested in 2016 (Guo et al., 2017). Despite their high economic value, phylogenetic relationships and taxonomy of *Pampus* species remain contentious. Based on 18 morphological characters, Liu et al. (2002) reconstructed the first phylogeny of *Pampus*. They suggested that *P. minor* was the early-branching species having fewer vertebrae and thicker otolith, whereas *P. punctatissimus* and *P. cinereus* were closely related sharing elongated dorsal and anal fin rays. Molecular studies using mitochondrial loci supported grouping *P. punctatissimus* and *P. chinensis* together (Cui et al., 2010; Divya et al., 2017), although morphologically

P. punctatissimus is more similar to *P. cinereus* and often referred to as “*P. punctatissimus* complex” (Liu and Li, 2013). However, existing molecular phylogenies of *Pampus* are based only on mitochondrial loci and their results are inconsistent (Cui et al., 2011; Cui et al., 2010; Divya et al., 2017). For example, *Pampus cinereus* was shown to be the earliest branch of *Pampus* (Divya et al., 2017), or grouped with *P. chinensis* and *P. punctatissimus*, or as polytomy with other species of *Pampus* (Cui et al., 2010). Nonetheless, mitochondrial loci are linked in vertebrates, and phylogenetic reconstruction based on mitochondrial data reflects gene genealogy, not necessarily species history. Moreover, many *Pampus* sequences lodged in GenBank have been found to be incorrect, probably due to species misidentifications (Divya et al., 2017).

The taxonomy of *Pampus* also has been unstable and is still highly debated due to morphological similarities between species

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<https://doi.org/10.1016/j.ympev.2019.106595>

Received 23 September 2018; Received in revised form 13 August 2019; Accepted 13 August 2019

Available online 14 August 2019

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Table 1Taxon sampling of 148 individuals of *Pampus* and three outgroup taxa.

Voucher number	Species	Collection site
CL1286-3, CL1287-2	" <i>Pampus liuorum</i> "	East China Sea, Shanghai, China (ES)
CL1303-1	" <i>Pampus liuorum</i> "	South China Sea, Guangxi province, China (SS)
CL1943-1, CL1943-2	" <i>Pampus liuorum</i> "	**
CL2010	" <i>Pampus liuorum</i> "	Bay of Bengal, Bangladesh (BB)
CL1285-1, CL1285-2, CL1285-3, CL1286-1, CL1286-2, CL1287-1, CL1287-3, CL1288-1, CL1288-3, CL1322-1, CL1343-1, CL1343-2, CL1343-3, CL1343-4,	" <i>Pampus echinogaster</i> "	East China Sea, Shanghai, China (ES)
CL1300-1, CL1300-13, CL1300-14, CL1300-16, CL1300-17, CL1300-18, CL1300-2, CL1300-3, CL1300-4, CL1300-5, CL1300-7, CL1300-8,	" <i>Pampus echinogaster</i> "	Bohai Sea, Liaoning province, China (BS)
CL1301-1, CL1301-10, CL1301-11, CL1301-12, CL1301-13, CL1301-15, CL1301-17, CL1301-2, CL1301-20, CL1301-21, CL1301-23, CL1301-24, CL1301-25, CL1301-3, CL1301-4, CL1301-5, CL1301-6, CL1301-7, CL1301-8, CL1301-9,	" <i>Pampus echinogaster</i> "	Bohai Sea, Shandong province, China (BS)
CL1303-2, CL1303-3, CL1305-2, CL1305-3, CL1345-4,	" <i>Pampus echinogaster</i> "	South China Sea, Guangxi province, China (SS)
CL1307-1, CL1307-10, CL1307-12, CL1307-13, CL1307-14, CL1307-15, CL1307-16, CL1307-17, CL1307-18, CL1307-19, CL1307-3, CL1307-4, CL1307-5, CL1307-6, CL1307-7, CL1307-8, CL1307-9,	" <i>Pampus echinogaster</i> "	Taiwan Strait, Fujian province, China (TS)
CL1944-1, CL1944-2, CL1944-3, CL1944-4, CL1944-5, CL1944-6, CL1944-7,	" <i>Pampus echinogaster</i> "	**
PKU57942	" <i>Pampus echinogaster</i> "	Mokpo-si, Jeollanam-do, Republic of Korea (K)
PKU59048	" <i>Pampus echinogaster</i> "	Gosan-ri, Hangeong-myeon, Jeju-si, Jeju-do, Republic of Korea (K)
PKU59394	" <i>Pampus echinogaster</i> "	Yeondo-ri, Nam-myeon, Yeosu-si, Jeollanam-do, Republic of Korea (K)
PKU59642, PKU59644	" <i>Pampus echinogaster</i> "	Jeollanam-do, Republic of Korea (K)
CL1288-2, CL1322-2, CL1944-8	<i>Pampus argenteus</i>	East China Sea, Shanghai, China (ES)
CL1300-10, CL1300-11, CL1300-12, CL1300-15, CL1300-19, CL1300-20, CL1300-6, CL1300-9, CL1301-14, CL1301-18, CL1301-19, CL1301-22	<i>Pampus argenteus</i>	Bohai Sea, Liaoning province, China (BS)
CL1305-1	<i>Pampus argenteus</i>	Bohai Sea, Shandong province, China (BS)
CL1307-2, CL1307-11	<i>Pampus argenteus</i>	South China Sea, Guangxi province, China (SS)
CL1304, CL1345-1, CL1345-2, CL1345-3, CL1345-5,	<i>Pampus argenteus</i>	Taiwan Strait, Fujian province, China (TS)
CL1976-1	<i>Pampus chinensis</i>	South China Sea, Guangxi province, China (SS)
CL1315-2, CL1316-2, CL1317, CL1318-1, CL1320-1, CL1320-2, CL2009	<i>Pampus chinensis</i>	**
CL1975-1, CL1975-2, CL1975-3, CL2012	<i>Pampus cinereus</i>	Bay of bangal, Bangladesh (BB)
CL1306-10, CL1306-4, CL1306-6, CL1306-7, CL1306-8, CL1306-9,	<i>Pampus cinereus</i>	**
CL1310-1, CL1310-15, CL1310-2, CL1310-20, CL1310-3, CL1310-4, CL1310-5,	<i>Pampus minor</i>	Taiwan Strait, Fujian province, China (TS)
CL1344-2, CL1843-1, CL1843-3, CL1843-4, CL1843-5, CL1943-3, CL1943-4, CL1943-5, CL1943-6,	<i>Pampus minor</i>	South China Sea, Guangxi province, China (SS)
PKU53770, PKU53772, PKU53774, PKU53776	<i>Pampus punctatissimus</i>	East China Sea, Shanghai, China (ES)
CL1323-1, CL1323-2	<i>Pampus punctatissimus</i>	Heuksan-myeon, Sinan-gun, Jeollanam-do, Republic of Korea (K)
CL1346-6	<i>Peprilus medius</i>	**
	<i>Psenopsis anomala</i>	South China Sea, Guangxi province, China (SS)

** Samples from fish market, unknown region.

(Supplementary Table S1). For example, there are five species of *Pampus*: *P. argenteus*, *P. chinensis*, *P. echinogaster*, *P. punctatissimus*, and *P. minor* currently listed in FishBase (Froese and Pauly, 2018), but three additional species: *P. cinereus*, *P. liuorum*, *P. nozawae* are included in the Catalog of Fishes (Eschmeyer et al., 2018) and in a recent research paper (Jawad and Liu, 2016). The most controversial issue in systematics of *Pampus* involves the identity of *P. argenteus* (Bleeker, 1852; Boeseman, 1947; Froese and Pauly, 2018; Haedrich, 1967; Li et al., 2013b; Li et al., 2017; Nakabo, 1993; Sun, 2015; Yamada, 1976) and the validity of *P. liuorum* (Liu and Li, 2013). Until now, limited molecular data have been collected to explicitly test whether *P. argenteus* and *P. echinogaster* are different species, or whether *P. liuorum* is valid or not (Cui et al., 2011; Cui et al., 2010).

In this study, we collected a total of 148 specimens of *Pampus* from the Bohai Sea, East China Sea, Taiwan Strait, South China Sea, the Korean coast, and Bay of Bengal, as well as three outgroup samples (Table 1). We collected data from 17,292 nuclear coding loci applying a targeted-gene enrichment method (Song et al., 2017) from a subset of 50 samples of all species of *Pampus* and two outgroup species (Supplementary Table S2). We sequenced cytochrome c oxidase subunit I (COI) for all 148 specimens of *Pampus* and analyzed them with sequences retrieved from the GenBank to evaluate the potential problem of misidentification and compare it with previous studies. We also re-examined external morphological characters of each sample and observed the osteological structure of each species using micro tomography (micro-CT). Our objectives were to (1) reconstruct a robust

phylogeny of *Pampus* using genome-scale nuclear loci, (2) investigate the validity of *P. argenteus* and *P. liuorum*, and (3) create a identification key for the genus of *Pampus*. Ultimately, our study was aimed to resolve the controversies about the phylogeny and taxonomy of *Pampus*, and provide a robust classification of *Pampus* for fisheries management.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Fin clips or muscle tissues were obtained from seven described species of *Pampus*: *P. echinogaster* (n = 80), *P. argenteus* (n = 18), *P. cinereus* (n = 11), *P. liuorum* (n = 6), *P. chinensis* (n = 6), *P. minor* (n = 13), and *P. punctatissimus* (n = 14). The species were identified according Liu et al. (2013a) and Liu and Li (2013). Each species was represented by more than five individuals from multiple regions (Table 1). All specimens were sampled directly from fishing vessels, except for three *P. cinereus* and two *P. liuorum*, which were collected from Dongfang Fish Market in Shanghai, China and one *P. cinereus*, which was collected from a fish market in Keelung, Taiwan, China (unknown origin). Two species, *Peprilus medius* (two individuals) and *Psenopsis anomala* (one individual), were used as outgroup taxa. 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. Target-gene enrichment, sequencing and read assembly

A subset of total samples of *P. echinogaster* ($n = 16$), *P. argenteus* ($n = 2$), *P. cinereus* ($n = 8$), *P. liuorum* ($n = 3$), *P. chinensis* ($n = 5$), *P. minor* ($n = 12$), and *P. punctatissimus* ($n = 4$) were carefully examined and used for target-gene enrichment. A suite of 17,817 single-copy conserved nuclear coding markers were used for target-gene enrichment (Song et al., 2017). The sequences of *Oreochromis niloticus* (3,508,171 bp) were used to design 120 bp biotinylated RNA baits (MYcroarray, Ann Arbor, Michigan). Library preparation and gene capture were carried out following Meyer and Kircher (2010) with some modifications (Li et al., 2013a) to accommodate capturing target loci of divergent species. Genomic DNA was sheared to about 250 bp using a Covaris M220 Focused-ultrasonicator (Woburn, Massachusetts, U.S.A.). Three hundred nanograms of genomic DNA were used for shearing and library preparation. Enriched and indexed products were pooled in equimolar ratios for sequencing on an Illumina HiSeq X10 lane at Annoroad Inc (Beijing, China).

Data processing and read assembly were performed according to description in Yuan et al. (2016). Reads from each sample were parsed according to their 8 bp index. Adapter sequences and low-quality reads (Qscore < 20) were trimmed using a custom Perl script (trim_adapter.pl, dryad xxx). PCR replicates were removed using a custom Perl script (rmrep.pl, dryad xxx), and then the reads were parsed to each gene file according to their similarity to target sequences of the reference species, *O. niloticus*. Trinity 2.2.0 (Grabherr et al., 2011) was used to perform initial read assembly with default parameters, and Geneious V7.1.5 (Kearse et al., 2012) was used to further merge assembled contigs. The assembled sequence that is most similar to the reference was selected according to Smith-waterman algorithm (Smith and Waterman, 1981). Finally, the selected contigs were compared to the genome of the reference using blast v2.2.27 (Camacho et al., 2009). The selected sequences were considered orthologous only if they had the best hit in the target region of the reference genome. The final assembled sequences that passed the orthology checking were further filtered based on quality and completeness of the data. The output amino acid (AA) sequences were aligned using MAFFT v7.369b (Katoh and Standley, 2013). The AA sequences were translated back to DNA after alignment. Pairwise distances of all loci were calculated using a custom Perl script (pdis.pl, dryad xxx) and loci with abnormally high distance values were realigned by eye or discarded if not correctable.

2.3. Phylogenetic analysis

The aligned DNA sequences were used for concatenated maximum likelihood (ML) analysis. Phylogenetic trees were estimated under a GTRGAMMA model partitioned by codon position with 1000 bootstrap replicates using RAxML v8.0.0 (Stamatakis, 2014). For comparison, data partitioning and ML tree reconstruction were carried out using IQtree v1.6.9 with the option “-m MFP: Extended model selection followed by tree inference” and 1000 ultrafast bootstrap (Nguyen et al., 2015). Bayesian analysis was performed using ExaBayes-1.4.1 with default settings (Aberer et al., 2014).

Individual gene trees were constructed with RAxML v8.0.0 under GTRGAMMA model. Gene trees were then used to generate a species tree with ASTRAL 4.10.6 (Mirarab et al., 2014). Two options “-q” “-t 2” were chosen to estimate the quartet score, the portion of input gene trees/quartet trees satisfied by the species tree. The resulting trees were visualized in FigTree v1.4.0 (Rambaut and Drummond, 2013).

2.4. Amplification of mitochondrial COI gene and analysis with sequences from GenBank

The mitochondrial COI gene was amplified and sequenced for all

151 samples (148 *Pampus* and 3 outgroup samples). Whole mitochondrial genome sequences of *P. argenteus*, *P. chinensis*, *P. cinereus*, *P. echinogaster* and *P. punctatissimus* were retrieved from GenBank and aligned, and transfer RNA sequences flanking the COI gene were selected for primer design, so the entire COI region could be amplified. Manually selected primers were checked with Primer3 web server (Rozen, 1996). The resulting left primer was 5'-CTACAACCCACCACT-TWCTCAGCCATC-3', and right primer was 5'-TGCAGAGYGGTTWT-GCGGTTGG-3'. PCR program included an initial preheating at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 66 °C for 30 sec, extension at 72 °C for 1 min, followed by a final extension for 7 min at 72 °C. Fish universal primers were used for amplifying outgroup species (Ivanova et al., 2007). The amplified products were sequenced at Sangon Biotech (Shanghai, China). Raw DNA sequences were edited manually using CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA). The 151 cleaned sequences were aligned with 155 COI sequences of *Pampus* downloaded from the GenBank database using MEGA7 (Kumar et al., 2016) (Supplementary Table S3). Maximum-likelihood (ML) and neighbor-joining (NJ) trees were reconstructed using MEGA7 to provide a graphical representation of divergence among species and to clarify species identification of sequences from GenBank database. The Tamura-Nei model was chosen in the ML analysis and evolutionary distances were computed using the Maximum Composite Likelihood method for NJ analysis (Kumar et al., 2016).

2.5. X-ray microtomography (micro-CT)

One specimen from each species of *P. argenteus*, *P. echinogaster*, *P. chinensis*, *P. cinereus*, *P. minor*, *P. punctatissimus* and *P. liuorum* was scanned using X-ray microtomography (voltage 60kv, current 280μA, optimum 2.88 μm; NSI X50, North Star Imaging, Inc., USA). Cross-sections of each specimen were reconstructed. The three-dimensional renderings were created, visualized, and manipulated with the program efx-CT (North Star Imaging, Inc., USA). By adjusting the gray value threshold, the image of bones remained while the soft tissues were virtually invisible.

2.6. Morphological measurements and establishment of the taxonomic key

Measurements were taken for *P. echinogaster* ($n = 60$), *P. argenteus* ($n = 17$), *P. cinereus* ($n = 5$), *P. liuorum* ($n = 6$), *P. chinensis* ($n = 1$), *P. minor* ($n = 3$), and *P. punctatissimus* ($n = 8$) following Haedrich (1967). A micro-CT was used to count the number of vertebrae (V), dorsal rays, (D) and anal rays (A). Total vertebrae was calculated as the sum of precaudal and caudal vertebrae (including urostyle). Length was measured as standard length (SL) and Body depth (BD), which was taken vertically at the origin of the first anal ray. Head length (HL) was measured from the upper lip to the posterior margin of the operculum. Eye diameter (ED) was taken as maximum length between the orbital rims. Predorsal distance (PDD) was measured from the tip of the snout to the base of the first dorsal ray. Length of pectoral fins (PL) was measured on the longest rays. The morphometric data were standardized as percentages of SL or HL. Gill rakers were counted from both the upper and lower limbs on the first gill arch. The shape of gill rakers (slender and needle-like; short and rounded) was recorded. Presence/absence of a groove on the lower ridge of gill cover was examined for each specimen. Coloration and pigmentation were documented in digital images of the specimens, which were stored in 70% ethanol after 10% formalin preservation. The formula of the interdigitation of anal-fin pterygiophores with the haemal spines was scored on micro-CT.

To investigate whether the standardized morphometric measurements of *P. liuorum* were affected by size, correlation between the ED/HL and the standard length, and between the PL/SL and the standard length were tested. Finally, we made a taxonomic key based on the results of molecular analyses and the morphological reexamination to

help species identification in the field.

3. Results

3.1. Sequencing results and assembly of gene-capture data

Illumina sequencing generated 336,731,746 raw reads, which is, 6,353,429 reads per sample on average (Supplementary Table S2). After adapter trimming and excluding low quality reads, there were 6,347,183 reads per sample. “*Pampus echinogaster*” (CL1300-4) had the least number of reads (1,821,364) and “*P. echinogaster*” (CL1343-1) had the most (33,350,322). When PCR duplicates were removed, 3,464,393 unique reads were left for each sample on average, accounting for more than 54% of the original reads. Loci with large average pairwise distances were checked by eye. After discarding loci that cannot be aligned confidently, 478,438 sequences were left, with an average of 9027 loci per sample. *Peprilus medius* (CL1323-1) had the fewest number of loci (1414) and *Pampus cinereus* (CL1320-1) had the most (15,900). Aligned sequences of concatenated loci were 3,294,747 bp with 190 bp per locus on average. The coverage for each locus was greater than 80% (< 20% missing data). The similarity between the targets and reference was greater than 60%. The number of parsimony-informative sites was 152,846 for the total alignment. (Supplementary Table S2).

3.2. Phylogenetic interrelationships between *Pampus* species based on genome-scale nuclear data

The concatenated ML analysis using RAxML and IQtree, and Bayesian analysis using ExaBayes all resulted in the same tree with all inter-specific nodes having a bootstrap support of 100 (Fig. 1, Figs. S1 and S2), except that *P. argenteus* were intermingled with *P. echinogaster*, whereas *P. liuorum* were mixed with *P. cinereus*. The species tree reconstructed using ASTRAL was also consistent with the concatenated trees (Fig. 2). The normalized quartet score of ASTRAL analysis was 0.44. Two major clades were supported by both the concatenated tree and the species trees, that one clade contained a sister group of *P. argenteus* and *P. minor*, and the other clade had *P. cinereus* sister to *P. chinensis* and *P. punctatissimus*.

3.3. Misidentification associated with sequence entries in GenBank revealed by COI analysis

Both NJ and ML analyses based on COI grouped all *Pampus* samples into five clades same as the NGS data (Supplementary Figs. S3 and S4). Only a priori identified *P. chinensis* had no sequences mixed with other species. The following samples were recovered in the *P. cinereus* cluster but were identified as *P. argenteus* (DQ107596-DQ107600, EU595224, EU752147-EU752148, FJ226531-FJ226533, KF192324-KF192325, KF192329, KF192335-KF192336, KF192341, KF202041-KF202050, KF373001-KF373012, KJ569773, KJ754096, KX530942-530949) and *P. sp.* (KM288588). The *P. punctatissimus* cluster included sequences likely misidentified as *P. cinereus* (KJ162.57, NC_022501, HM107697-HM107699) and the *P. minor* cluster contained sequences identified as *P. argenteus* (EF607457-EF607460) and *P. cinereus* (EF607461-EF607466). The *P. argenteus* cluster was composed of *P. argenteus*, *P. echinogaster* (NC_023258, KF373560, KJ539194, JN242665-JN242670, KJ539185, KJ539197) and *P. sp.* (FJ434342-FJ434343, FJ652423-652427, EU357803, NC_011707). The sequences of *P. argenteus* and *P. echinogaster* collected in this study were also mixed together. *Pampus cinereus* and *P. liuorum* did not form reciprocally monophyletic groups.

3.4. Morphological analyses and a key for species identification

The number of vertebrae, the number of spines and rays, the number of pterygiophores, and the formula for the interdigitation of

anal-fin pterygiophores with the haemal spines for each species can be found in Table 2. Other morphometric measurements and meristic counts for all specimens are listed in supplementary Table S4. The morphometric characters used to distinguish *P. liuorum* from *P. cinereus* (ED/HL and PL/SL) were checked. Both characters were significantly correlated with standard length, decreasing in larger fish (Fig. 3a for PL/SL, $R^2 = 0.43$, $p < 0.01$; Fig. 3b for ED/HL, $R^2 = 0.58$, $p < 0.01$). Gill rakers of *P. cinereus* and *P. liuorum* were short and rounded, but those of all other species were slender and needle-like (Supplementary Fig. S5). The groove on the lower ridge of gill cover was only observed in *P. argenteus* and *P. echinogaster* (Supplementary Fig. S6).

Key to species of *Pampus*

- 1a. Mouth terminal, forehead almost straight. Some jaw teeth having three cusps. Body very deep: equal to standard length minus head length ... *P. chinensis* (Euphransen, 1788).
- 1b. Mouth terminal, forehead not straight, upper jaw protrudes over lower jaw slightly. Jaw teeth with no branched cusps. Body depth shorter than the standard length minus head length.....go to 2
- 2a. Gill rakers, short and rounded. *P. cinereus* (Bloch, 1795). [syn. *P. liuorum* Liu and Li, 2013].
- 2b. Gill rakers slender and needle-like..... go to 3
- 3a. Groove on the lower ridge of gill cover extending under lower jaw ... *P. argenteus* (Euphransen, 1788). [syn. *P. echinogaster* (Basilevsky, 1855)].
- 3b. Groove on the lower ridge of gill cover absent..... Go to 4
- 4a. Patch of longitudinal sensory canals that under lateral line extends above pectoral-fin base or beyond. Vertebrae: 34 *P. punctatissimus* (Temnick, 1845).
- 4b. Patch of longitudinal sensory canals that under lateral line extends shortly, not reach base of pectoral-fin base. Vertebrae: 29–30 *P. minor* Liu and Li, 1998.

4. Discussion

4.1. Phylogenetic interrelationships of *Pampus*

Studies using mitochondrial data converged on the conclusion that *P. argenteus* and *P. minor* were sister species, but the phylogenetic position of *P. cinereus* was inconsistent among studies. *Pampus cinereus* was shown to be the earliest branch of *Pampus*, or grouped with *P. chinensis* and *P. punctatissimus*, or as polytomy with other species of *Pampus* (Cui et al., 2010; Divya et al., 2017). Our gene-capture experiments generated data from thousands of nuclear loci, which may be used to trace the species history instead of just a single gene genealogy. The results of phylogenetic analyses based on gene-capture data are consistent among different analytical approaches. Our concatenated gene tree and species tree analyses using the genome-scale nuclear data resulted in identical and highly supported phylogeny, in which *P. argenteus* is sister to *P. minor* in one clade, and *P. cinereus* sister to *P. chinensis* and *P. punctatissimus* in the other clade. The shared appearance of elongated dorsal and anal fin between *P. cinereus* and *P. punctatissimus* may be due to convergent evolution.

Divya et al. (2017) reported that *Pampus* sp. collected from the Indian Ocean are very different from *P. argenteus*. Instead they were closely related to *P. cinereus* from China, but formed distinct clades. Our analyses also found three clades grouped according to localities (Fig. 1). Further range-wide sampling for morphological and molecular study is under way to describe potential cryptic species in *P. cinereus*.

4.2. Identity of COI sequences of *Pampus* in Genbank

The phylogenetic trees based on COI sequences collected by us and those retrieved from Genbank indicate that many sequences from Genbank are mislabeled. Some of these errors may be due to

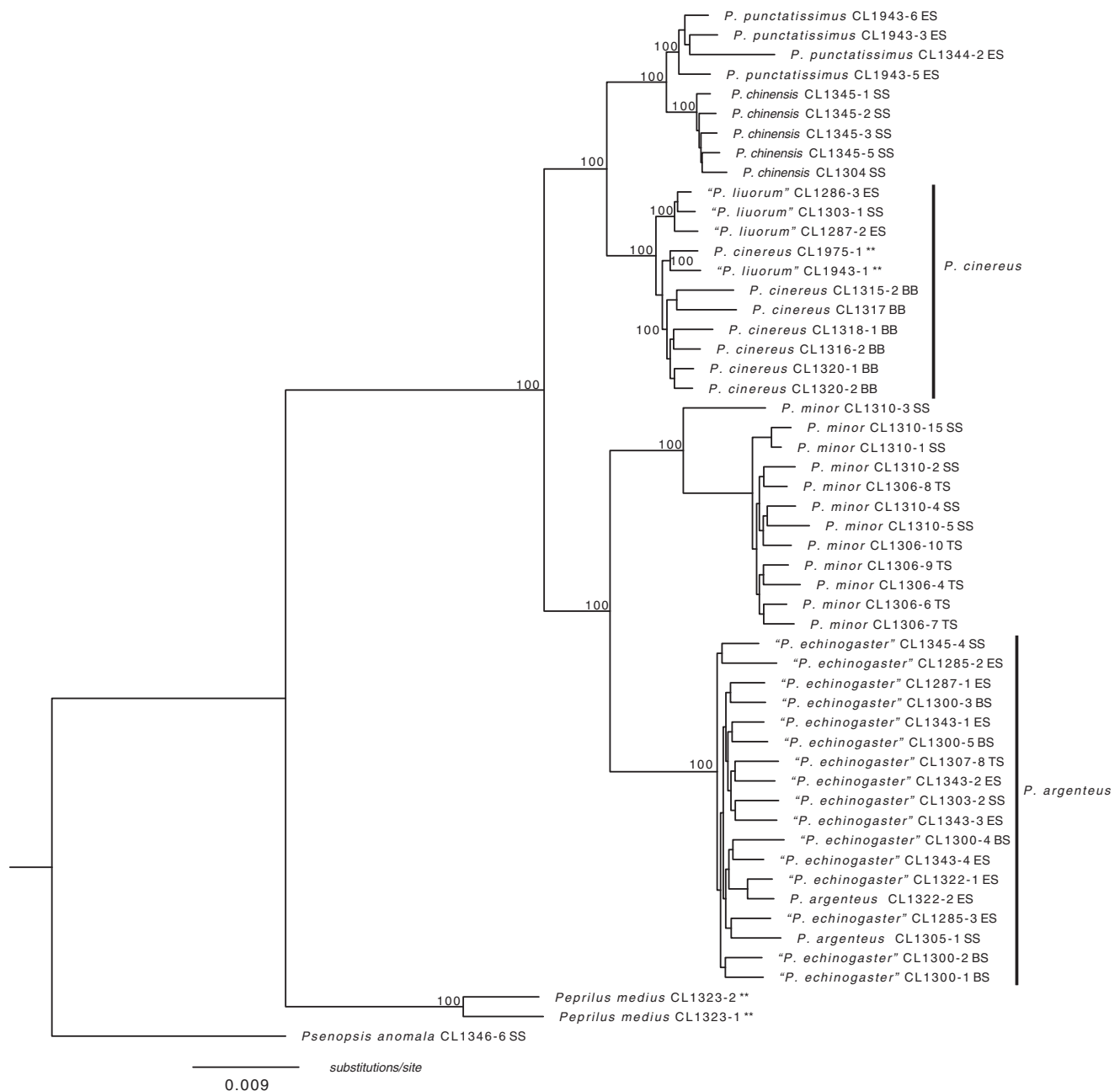


Fig. 1. Concatenated ML tree of *Pampus* based on sequences of 17,292 loci. Bootstrap support values are 100 for all nodes at or above species level. The "species" in quote are junior synonyms or invalid but currently used in some literature. ES, the East China Sea; SS, the South China Sea; BB, the Bay of Bengal; TS, the Taiwan Strait; BS, the Bohai Sea. The asterisks indicate samples bought from the fish market with unknown origins.

misidentification. For example, *P. punctatissimus* is often confused with *P. cinereus* because both species have elongated dorsal and anal fin rays. Adult *P. minor* is often confused with young *P. argenteus* or *P. cinereus* as they have a similar appearance. Other errors may be due to taxonomic uncertainty of *Pampus* species. For example, *P. argenteus* and *P. echinogaster* are indistinguishable and their names are used interchangeably, and naming *P. cinereus* as *P. argenteus* still is favored by some authors (Froese and Pauly, 2018; Li et al., 2013b). The erroneous *Pampus* entries in Genbank suggest that caution should be taken when working with data from Genbank, particularly in this group of fishes. Applying the identification key established in this study to validate the voucher specimens associated with the sequences in GenBank may help to clarify the issue.

Phylogenetic trees based on mitochondrial genes were unstable and contradictory between our results (Figs. S3 and S4) and in previous

studies (Cui et al., 2011; Cui et al., 2010; Divya et al., 2017). *Pampus cinereus* was shown to be the earliest branch of *Pampus* (Fig. S4) (Divya et al., 2017), or grouped with *P. chinensis* and *P. punctatissimus*, (Fig. S3) (Cui et al., 2010). This may be due to different taxon sampling and insufficient character sampling. For example, the length of COI sequence was 584 bp, with 176 phylogenetic informative sites in our dataset.

4.3. *Pampus echinogaster* is a synonym of *P. argenteus*

The COI gene tree and the concatenated nuclear gene tree both suggest that *P. argenteus* and *P. echinogaster* are one species (Supplementary Figs. S3 and S4 and Fig. 1). Because *P. argenteus* was described in 1788 and *P. echinogaster* was described in 1855, we consider *P. argenteus* as the valid species name and *P. echinogaster* as a

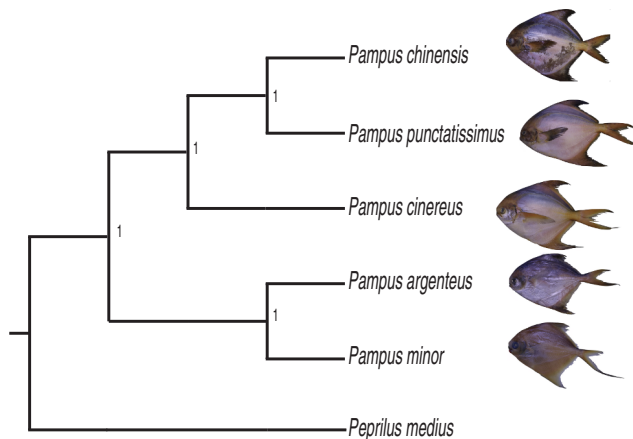


Fig. 2. The species tree obtained from ASTRAL analysis using 17,292 loci. All nodes have a branch support value (default in ASTRAL) of 1.

synonym. Liu et al. (2013a) stated that *P. argenteus* could not be found northward of the South China Sea and *P. echinogaster* was not distributed in the South China Sea. Recently, we collected *P. argenteus* from the South China Sea (type locality), the East China Sea, upward to the Bohai Sea and “*P. echinogaster*” from the Korean coast, the Bohai Sea (type locality), the East China Sea and downward to the South China sea, thus, further supporting that the two “species” should be only one species.

Pampus echinogaster and *P. argenteus* have been considered distinct by a number of authors (Haedrich, 1967; Li et al., 2013b; Nakabo, 2002), but their morphological descriptions were inconsistent. The original description of *P. echinogaster* (Basilewsky, 1855) did not include meristic counts and only described the general morphology, so it is difficult to compare morphology for this species with that of others. Haedrich (1967) gave a dorsal fin count of V-X, 38–43, an anal fin count of V-VII, 34–43 and 14–16 + 20–25 vertebrae for *P. argenteus*, and D VIII-IX 42–49, A V-VII 42–47 and 14–16 + 24–26 vertebrae for *P. echinogaster*. Compared with Euphrasen's original morphologic description of *P. argenteus*, *P. echinogaster* described by Haedrich (1967) should be *P. argenteus* according to its number of fin rays, but the *P. argenteus* he described is potentially another species. In a redescription of *P. argenteus*, Liu et al. (2013a) gave counts of D IX-XI 44–48, A VI-VII 43–47 and 16 + 24 vertebrae, which overlapped with their counts of D IX-XI 44–49, A VI-VII 42–48 and 16 + 24–25 vertebrae for *P. echinogaster*. They distinguished the two species primarily on the basis of body depth, pre-dorsal length, pre-anal length, snout length, and vertebral count (40 vs 40–41), all of which were reported to be greater in *P. echinogaster*. In a later paper, Jawad and Liu (2016) reported a reverse pattern of vertebral count, *P. echinogaster* (39) vs. *P. argenteus* (41). We counted the number of vertebrae of *P. argenteus* and *P. echinogaster* as

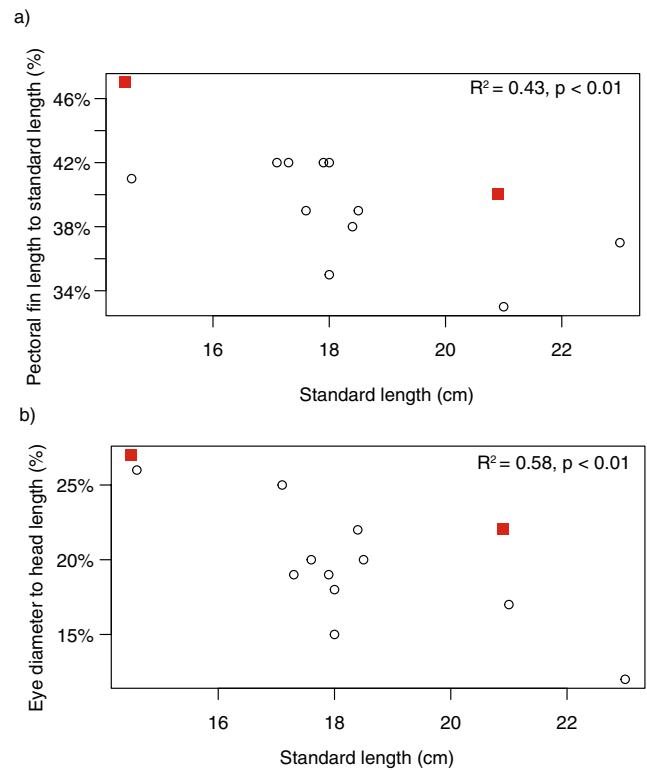


Fig. 3. Correlation between the pectoral fin length (divided by the standard length) and the standard length (a); and between the eye diameter (divided by the head length) and the standard length (b). Squares are type specimens: *Pampus liuorum* (left, holotype, Liu and Li, 2013) and *P. cinereus* (right, neotype, Liu et al., 2013).

41 and 39–40 respectively (Table 2), suggesting that vertebral counts should not be used to distinguish these two species.

4.4. *Pampus argenteus* and *P. cinereus* are two distinct species

Pampus argenteus originally described by Euphrasen (1788) based on a specimen from Guangdong province, China, was characterized as having a dorsal fin with 9 min spines and 45 rays, and an anal fin with 7 min spines and 43 rays. Some author think *P. cinereus* as a synonym of *P. argenteus* (Froese and Pauly, 2018; Haedrich, 1967; Li et al., 2013b; Li et al., 2017; Sun, 2015), whereas others accept *P. cinereus* as a valid species (Cui et al., 2011; Cui et al., 2010; Divya et al., 2017; Liu et al., 2013b). *Pampus cinereus* has fewer dorsal fin rays (37–41 vs 45) and anal fin rays (36–41 vs 43) compared to the original description of *P. argenteus* (Liu et al., 2013a) (Table 2). Our molecular results also show that *P. argenteus* and *P. cinereus* are two distinct species (Figs. 1 and 2) and should be treated as such.

Table 2
Osteological characters of seven “species” of *Pampus*.

Voucher number	Species	No. of vertebrae	No. of spines	No. of rays	No. of pterygiophores	The formula for the interdigitation of anal-fin pterygiophores with haemal spines
CL1944-2	“ <i>P. echinogaster</i> ”	40	6	42	45	7P-C-2P-2P-2P-2P-2P-2P-3P-2P-2P-3P-2P-2P-2P-2P-2P-2P-2P-2P
CL1976-1	<i>P. chinensis</i>	32	6	40	37	9P-C-2P-2P-3P-3P-3P-2P-2P-3P-2P-4P-2P
CL1975-3	<i>P. cinereus</i>	37	5	40	41	7P-C-2P-2P-1P-2P-3P-2P-2P-2P-3P-2P-2P-3P-2P-2P-2P
CL1300-9	<i>P. argenteus</i>	41	9	46	50	6P-C-1P-1P-1P-1P-2P-1P-1P-2P-2P-3P-3P-4P-2P-2P-2P-3P-2P-2P-2P-2P-2P-2P-2P-P
CL1286-3	“ <i>P. liuorum</i> ”	38	6	38	39	10P-C-2P-2P-2P-2P-2P-3P-2P-2P-2P-2P-2P-2P-1P-2P-1P
CL1306-10	<i>P. minor</i>	29	6	36	37	6P-C-2P-4P-3P-3P-2P-3P-3P-2P-3P-2P-1P
CL1843-4	<i>P. punctatissimus</i>	35	6	39	42	8P-C-2P-2P-2P-2P-3P-3P-2P-2P-2P-2P-2P-2P-4P-2P-2P

C, haemal spine of the 1st caudal vertebra.

P, pterygiophores.

4.5. *Pampus liuorum* is not valid

Pampus cinereus was originally described by Bloch (1795). This species had a greatly extended anal fin and long pectoral fins. *Pampus liuorum* was proposed by Liu and Li (2013) using specimens from the Zhuhai fish market, Guangdong, China. The vertebral count of *P. liuorum* was 38; D. VII-IX, 37–41; A. IV-VI, 36–40; P. 22–24. It differed from *P. cinereus* by having more total vertebrae (38 vs. 36), a shorter pectoral-fin length (31.5–41.5% vs. 42.0–47.2%), and shorter eye diameter (19.4–25.0% vs. 27.7–30.7%). Jawad and Liu (2016) counted the same number of vertebrae (37) for both species in a recent paper. Our counts for the number of vertebrae were 36–37 for *P. cinereus* and 36–38 for *P. liuorum* (Table 2), suggesting that vertebra counts cannot be used to distinguish *P. cinereus* and *P. liuorum*. The other two discriminating characters proposed by Liu and Li (2013), the ratio of eye diameter to head length and the ratio of pectoral fin length to standard length both were significantly correlated with the size of the fish ($p < 0.01$; Fig. 3). Furthermore, neither mitochondrial data nor genome-scale nuclear data supported *P. liuorum* as a valid species (Supplementary Figs. S3 and S4 and Fig. 1). Thus, all characteristics of *P. liuorum* are non-definitive, and *P. liuorum* should be synonymous to *P. cinereus*.

5. Conclusions

We reconstructed a robust phylogeny of the economically important fish genus, *Pampus*. We found that *P. argenteus* is sister to *P. minor* in one clade, and *P. cinereus* sister to *P. chinensis* and *P. punctatissimus* in the other clade. *Pampus cinereus* is neither the earliest branching *Pampus* species, nor the sister species to *P. punctatissimus* suggested by their appearance. We found that *P. echinogaster* is a synonym of *P. argenteus* and *P. liuorum* is not valid, based on both morphological and molecular data. More samples from the whole distribution of *P. cinereus* should be collected to investigate the evolutionary history of this fish and potential “cryptic” species. We created a convenient identification key for the genus of *Pampus*.

6. Data availability

The raw fastq reads are available in NCBI repository with accession number, PRJNA490771, and the COI sequences with accession number MK893273–MK893402. Sequences alignment of nuclear loci and other data file can be found in the online version of this article, at <https://doi.org/10.1016/j.ympev.2019.106595>.

Acknowledgements

We thank Mr. Jianhong Xia of Shanghai Natural History Museum for his thoughtful comments and edits for the manuscript. We thank Mr. Calder Atta from the University of Washington for his help with English writing. This research was supported by the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, and Science and Technology Commission of Shanghai Municipality (19050501900) to CL. The Shanghai Oceanus Supercomputing Center (SOSC) provided computational resource for data analyses. Micro-CT Laboratory of the Shanghai Nature History Museum provided X-ray microtomography data.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2019.106595>.

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