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Morphological characteristics and DNA barcoding of *Pampus echinogaster* (Basilewsky, 1855)

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Abstract

The morphological similarities of *Pampus* fishes have led to considerable confusion in species-level identification, and no accurate information on neotype or DNA barcoding of *Pampus echinogaster* is available. Two hundred and seven specimens of *P. echinogaster* were collected from the coastal waters of Dandong, Dongying, Qingdao, Nantong, Zhoushan, Wenzhou, Changle, Taiwan, and Wakayama (Japan), from June 2010 to April 2013. The diagnostic characteristics of *P. echinogaster* are as follows: dorsal fin VIII-XI-43–51, anal fin V-VIII-43–49, pectoral fin 22–27, caudal fin 19–22, pelvic fin absent; first gill rakers sparse, slender (pointed), 3–4+12–16=15–20; vertebrae 39–41; transverse occipital canal on top of head moderately small, wavy ridges not reaching upper origin of pectoral fin; ventral branch of lateral line canal spare, shorter than dorsal branch of lateral line canal. By combining congener sequences of the cytochrome oxidase I (COI) gene from GenBank, two absolute groups were detected among all specimens, which further indicated that two valid species were present based on genetic differences in amino acid sequences and the distance between the groups. The sequences of Group 1 can be regarded as DNA barcoding of *P. echinogaster*. The correct morphological redescription and DNA barcoding of *P. echinogaster* are presented here to provide a guarantee for efficient and accurate studies, a theoretical basis for classification, and enable appropriate fishery management and conservation strategies for the genus *Pampus* in the future.

Key words: morphological characteristics, DNA barcoding, *Pampus echinogaster*, genetic differentiation, transverse occipital canal

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1 Introduction

The genus *Pampus* (Perciformes: Stromateidae) has six valid species worldwide (Liu and Li, 2013): *Pampus echinogaster*, *P. argenteus*, *P. chinensis*, *P. cinereus*, *P. punctatissimus* and *P. minor*. These commercially important species are widely distributed in the coastal waters of China and the Indo-western Pacific (Liu and Li, 2013). The morphological similarities of *Pampus* fish have led to considerable confusion in species-level identification. Numerous previous investigations of the genus have been conducted (Liu and Li, 1998, 2013; Cui et al., 2010; Guo et al., 2010), with *P. argenteus* being the species most widely studied (Peng et al., 2009; Zhao et al., 2011; Sun et al., 2013). The most unknown species is *P. echinogaster*. Furthermore, no morphological descriptions or regional fauna reports in China appear to correspond to a valid species: where is *P. echinogaster* found? After some investigation, we hypothesized that the well-studied *Pampus* species of China is not *P. argenteus* but *P. echinogaster* and that *P. argenteus* is a second valid species that is distributed southward from the Taiwan Strait.

The mitochondrial cytochrome oxidase I (COI) gene varies noticeably among species and very little among individuals of the same species. The use of a fragment of the COI gene for DNA barcoding (Hebert et al., 2003a) has been extremely effective and can help expand our knowledge by discriminating among species (Domingues et al., 2013; Puckridge et al., 2013), discovering newly recorded and new species (Qin et al., 2013; Xiao et al., 2016), revealing cryptic species (Hajibabaei et al., 2007; Zemlak et al., 2009), and identifying ichthyoplankton (Ko et al., 2013; Hubert et al., 2015), which can also be sequenced with universal primers. The COI gene enables accurate animal species identification only when adequate reference sequence data are available. However, misidentified DNA barcoding of the *Pampus* genus has been found in GenBank (Cui et al., 2010; Guo et al., 2010; Li et al., 2013), and correct identifications are needed. The first objective of the present study is to investigate the species validity of *P. echinogaster* based on morphological and molecular evidence. The second objective is to describe this species based on accurate morphological characteristics and DNA barcoding. Finally, the third objective is to correct the current COI sequences of this species released on GenBank.

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For further investigation, *Pampus* fish were collected from the coastal waters of China and Japan from June 2010 to April 2013. The results highlight the need for caution when identifying species in the *Pampus* genus from China and will facilitate the fishery management, biodiversity conservation, and sustainable exploitation of this species.

2 Methods

2.1 *Sample collection*

Samples were collected from Dandong, Dongying, Qingdao, Nantong, Zhoushan, Wenzhou, Changle, Taiwan and Wakayama (Japan), from June 2010 to April 2013 (Fig. 1, Table 1). In addition, two *P. chinensis* individuals were collected as outgroup for the present study. All individuals were identified based on morphological characteristics (Nakabo, 2002; Yamada et al., 1986, 2009), and a piece of muscle tissue was obtained from each individual and preserved in 95% ethanol. All examined specimens were frozen and preserved at –20°C.

2.2 *Morphological analysis*

Counting and measurement methods were performed as described by Elliott et al. (1995) with some modifications. The counts included the following characteristics: dorsal fin spines and rays, pectoral fin rays, anal fin spines and rays, caudal fin rays, gill rakers on the first gill arch, and vertebrae. The measurements included the following traits: standard length, fork length, head length, postorbital length, snout length, eye diameter, interorbital width, caudal peduncle depth, caudle peduncle length, body depth, and body width. All measurements were performed using calipers to the nearest 0.1 mm. Color and pigmentation were documented in fresh fish, and all remaining measurements were obtained using performed specimens.

2.3 *Molecular analyses*

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method. The fragment of mitochondrial DNA COI was amplified using the primers F1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′; and R1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ (Ward et al., 2005). Each polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 17.5 μL of ultrapure water, 2.5 μL of 10×PCR buffer, 2 μL of dNTPs, 1 μL of each primer (5 μmol/L), 0.15 μL of Taq polymerase, and 1 μL of DNA template. PCR amplification was performed in a Biometra thermal cycler under the following conditions: 5 min of initial denaturation at 95°C; 30 cycles of 45 s at 94°C for denaturation, 45 s at 52°C for annealing, and 45 s at 72°C for extension; and a final extension at 72°C for 10 min. The PCR products were purified with a Gel Extraction Mini Kit. The purified product was used as the template DNA for cycle sequencing reactions performed using the BigDye Terminator Cycle Sequencing Kit, and bi-directional sequencing was conducted on an ABI Prism 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification.

To determine the correct DNA barcoding of *P. echinogaster*,

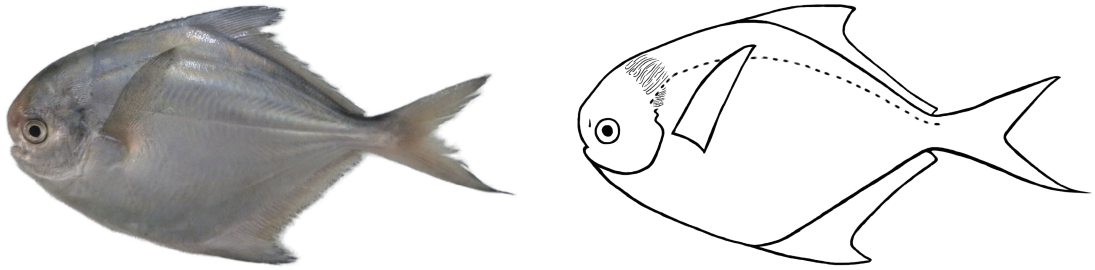

Fig. 1. *Pampus echinogaster* (Basilewsky, 1855).

Table 1. Information on the *Pampus* samples and sequences in this study

Species	This study				Cited accession No.
	Sampling site	Sampling time	Number	Accession No.	
P. echinogaster	Dandong	Jun. 2010	DD1-DD2	KJ539196, KJ539197	JN242665, JN242666, JN242667, JN242668, JN242669, JN242670
	Dongying	Sep. 2011	DY1-DY2	KJ539180, KJ539181	
	Qingdao	Sep. 2011	QD1-QD2	KJ539184, KJ539185	
	Nantong	Jun. 2012	NT1-NT2	KJ539182, KJ539183	
	Zhoushan	Apr. 2012	ZS1-ZS2	KJ539192, KJ539193	
	Wenzhou	Aug. 2009	WZ1-WZ2	KJ539190, KJ539191	
	Changle	Apr. 2013	CL1-CL2	KJ539194, KJ539195	
	Taiwan	Aug. 2004	TW1-TW2	KJ539186, KJ539187	
	Wakayama	Jan. 2013	WK1-WK2	KJ539188, KJ539189	
Pampus sp.					FJ652426, FJ652427
P. argenteus	Beibu Gulf	Dec. 2011		KF192324	Q738583, JQ738584, JQ738585, JQ738586, JQ738587, JQ738588, HM068249, HM068250, HM068251, HM068252, HM068253, HM068254, HM068255, HM068256, HM068257, HM068258, HM068259, HM068260, HM068261, HM068262, HM068263, HM068264
	Taiwan Island	Sep. 2012		KF192337	
	Kuwait	Sep. 2011		KF192325	
	Pakistan	Dec. 2010		KF192332	
	Bay of Bengal				FJ384702
P. chinensis	Beibu Gulf	Dec. 2011			KF192330, KF192331

COI sequences were downloaded from GenBank for comparative analysis (Table 1). The sequences were aligned using DNASTAR software (Madison, WI, USA). A neighbor-joining (NJ) tree was created, and the distances between and within groups were calculated using MEGA 5.0 (Tamura et al., 2011) with 1 000 bootstrapping replications based on evolutionary distances calculated using the best selected K2P model.

3 Results

3.1 *Morphological analysis*

Counts and measurements from 207 *P. echinogaster* individuals were conducted. The standard length ranged from 82 to 188 mm, and fork length ranged from 91 to 200 mm. This species can be distinguished from all congeners using the following combination of characteristics:

Measurements presented as percentages of standard length (%): head length 15.5–27.4, fork length 106–124, dorsal fin length 16.8–22.5, anal fin length 21.4–29.3, pectoral fin length 30.1–36.2, body depth 51.2–60.9, body width 8.5–15.8. Measurements presented as percentages of head length (%): snout length 16.2–23.5, eye diameter 23.9–26.8, interorbital width 35.8–47.8, postorbital length 48.2–59.4, caudal peduncle depth 13.2–19.4. Measurement is a percentage of the caudle peduncle length (%): caudal peduncle depth 25.2–38.2.

Body oval-shaped, compressed, covered by small deciduous scales. Dorsal and ventral profile strongly keeled, the base of dorsal fin highest. Head small, compressed. Snout blunt and short, forehead arched slightly, equal to or slightly shorter than eye diameter. Mouth small, subterminal, slit curved downward posteriorly, reaching middle of eye, not moveable. Lower jaw shorter than upper. Eyes small, up and front of head. Teeth on the jaw minute, a single jaw, compressed from the sides, absent on the vomer and platinum. Gill membranes joined to belly, lower trench of gill membrane long, first gill rakers sparse, slender (pointed), 3–4+12–16=15–20. Vertebrae 39–41.

Dorsal fin VIII-XI-43–51, anal fin V-VIII-43–49, anterior-most rays of the median fins often produced and formed into a falcate lobe, followed by shorter rays, originating behind pectoral fin bases, anterior spine of median fins distinct in juveniles, reduced and embedded in skin with growth. Pectoral fin 22–27, long, reaching the middle of the dorsal fin base. Caudal fin 19–22, moderately long, deeply forked, lower lobe slight extended. Pelvic fin absent.

Scales very small, cycloid and deciduous, covering body, extending onto bases of all fins, absent on jaws and snout. Lateral line complete, high, following dorsal profile to caudal peduncle, arched, parallel with back rim. Transverse occipital canal on the top of head moderately small, wavy ridges not reaching upper origin of pectoral fin. Ventral branch of lateral line canal spare, shorter than dorsal branch of lateral line canal.

Color bluish and gray on the back and silvery white on ventral sides. Anterior rays of dorsal and anal fins dark gray, and posterior part of dorsal and anal fins flesh color with pale gray rim. Caudal fin pale gray with dark posterior rim. Pectoral fins gray with some small black dots.

3.2 *Molecular analyses*

The COI gene fragments of two *P. echinogaster* individuals randomly chosen from each population were sequenced. All COI sequences were deposited in GenBank under the accession numbers KJ539180–KJ539197. A set of "*P. argenteus*" and *Pampus* sp. sequences were downloaded from GenBank and total 53 sequences were employed with a length of 625 bp in the analysis at last. In total, one hundred and six variable sites, one hundred and three parsimony-informative sites, and three singleton sites were identified by sequence comparison, and no deletions or insertions were observed. The alignments of all the sequences revealed that most sequences had the highest similarity to *P. echinogaster* with the fewest variable sites. The base frequencies were heterogeneous across all taxa for all three codon positions. The A+T content (57.4%) was higher than that of G+C (42.6%). Strong compositional biases against G in the third position were observed.

An NJ tree was constructed based on the K2P model with 1 000 replications of the bootstrapping test. *Pampus chinensis* was chosen as outgroup to root the tree (Fig. 2). As shown in the NJ tree, two groups were formed, excluding the outgroup. Forty-eight sequences clustered within Group 1. Within Group 1, all of the *P. echinogaster* and most of the "*P. argenteus*" individuals clustered together, indicating a closer relationship with each other than with either Group 2 or *P. argenteus*. Group 2 included five sequences that included other "*P. argenteus*" individuals. Both JN242665–JN242670 and *Pampus* sp. clustered with our *P. echinogaster* sequences in Group 1.

In the protein-coding COI gene fragments, mutations in the third (and rarely in the first) position of codons that did not result in amino acid substitutions (most were silent or synonymous substitutions) accumulated much more rapidly than did amino acid replacement substitutions (non-synonymous substitutions). The most frequently observed substitutions were transitions in the third position of codons; the second-most frequent substitutions were transversions in the third positions and silent transitions in some first codon positions (Meyer, 1993). A total of 207 amino acids were translated from the 625 base pairs. Only one amino acid mutation was observed in Group 1 and Group 2, respectively. Four amino acid mutations were detected between the two groups. Therefore, some divergence occurred at the amino acid level.

Based on the K2P model, the genetic distances of COI within and between the two groups were computed (Table 2). The mean genetic distance within Group 1 was 0.002, in contrast to the distance of 0.004 within Group 2. The evolutionary distance between Group 1 and Group 2 was 12.6%, far exceeding the threshold of species delimitation (approximately 2%) (Hebert et al., 2003b).

4 Discussion

Until 1905, the *Pampus* genus was widely accepted as distinct from the *Stromateus* genus, which was first proposed by Bonaparte in 1837. Nowadays, most ichthyologists believe that *Pampus* should be divided into six species: *P. argenteus*, *P. echinogaster*, *P. punctatissimus*, *P. cinereus*, *P. chinensis* and *P. minor* (Yamada et al., 2009; Cui et al., 2010; Liu and Li, 2013). Recently, a new *Pampus* species, *Pampus liuorum*, was reported by Liu and Li (2013). *Pampus* species are important commercial species that are widely distributed in the Indo-western Pacific. However, species boundaries among highly similar or variable sibling species remain to be clarified. The morphological similarities and complexities of *Pampus* species have led to taxonomic confusion with regards to nomenclature. Within the genus, the most mysterious species is *P. echinogaster*, for which no reports of the neotype are available. Where is *P. echinogaster* species found? Is it a valid species?

Li et al. (2013) redescribed *P. argenteus* based on its morphological characteristics and DNA barcoding and determined that

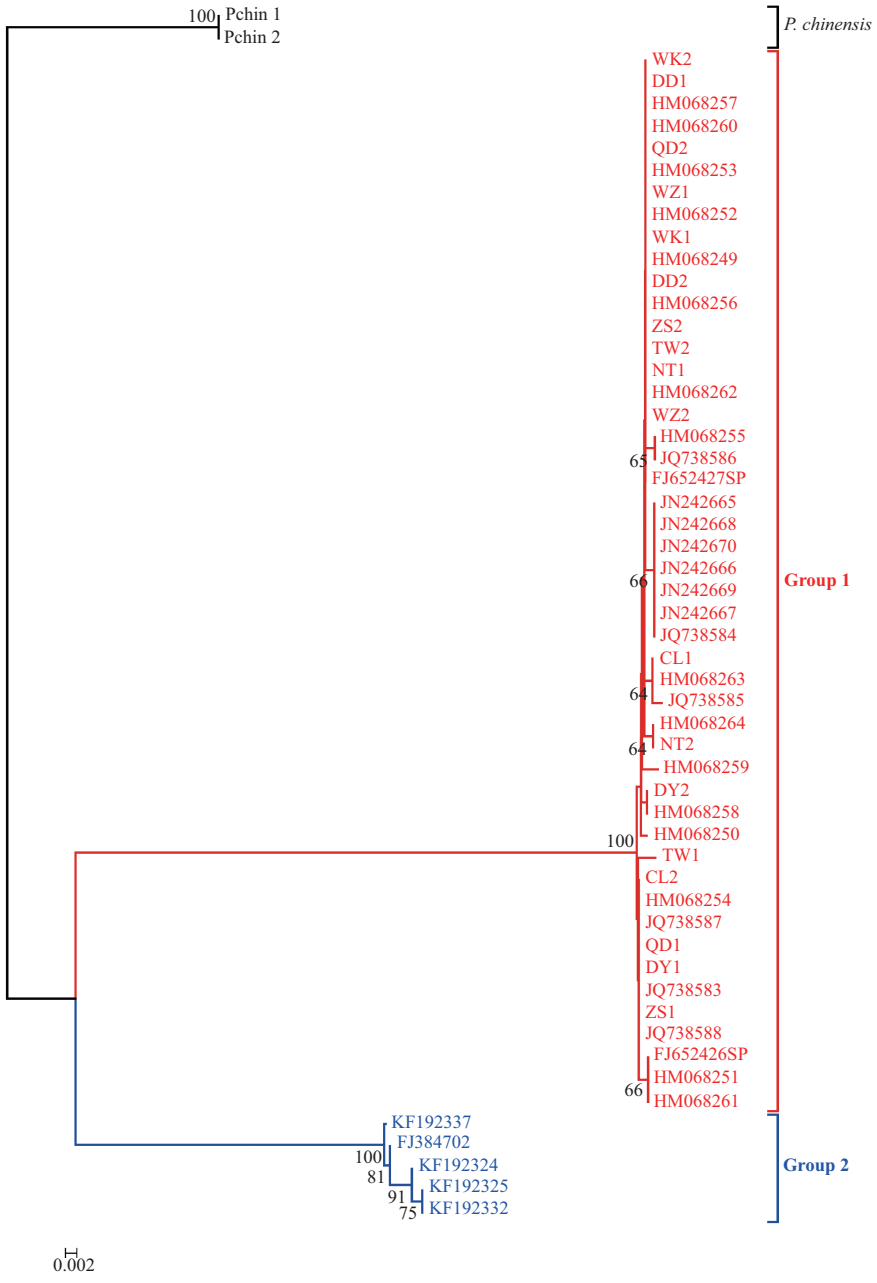

Fig. 2. Neighbor-joining (NJ) tree constructed using the K2P model for COI gene sequences of *P. echinogaster*. Bootstrap values of being greater than 50% from 1 000 replicates are shown.

Table 2. Genetic distances of COI within (on the diagonal) and between (below the diagonal) groups

	Group 1	Group 2	*P. chinensis*
Group 1	0.002		
Group 2	0.126	0.004	
P. chinensis	0.148	0.114	0.00

the so-called "*P. argenteus*" distributed in the Bohai Sea, Yellow Sea and East China Sea was not *P. argenteus*. We doubt the validity of all morphological records of *P. argenteus* in Chinese taxonomic books (Cheng, 1962; Zhu et al., 1963; Yang and Cheng, 1987; Zhang et al., 1994; Li, 1998). Similar doubts have been raised by some foreign ichthyologists (Nakabo, 2002; Dolganov et al., 2007; Yamada et al., 2009). The morphological characteristics of *P. echinogaster* in this study are apparently different from those of the *P. argenteus* described by Li et al. (2013) and resemble those of "*P. argenteus*" and "*P. echinogaster*" according to Liu et al. (2013) (Table 3). Thus, we conclude that the records of "*P. argenteus*" in China are actually those of *P. echinogaster*, consistent with the results of Li et al. (2013). Unfortunately, the original description of *P. echinogaster* by Basilewsky was far from satisfactory, and modern ichthyologists have been unable to locate its type specimens (Abe and Kosakai, 1964); Furthermore, most morphological descriptions of this species in Chinese taxonomic books either overlap or span the species range (Cheng, 1962; Zhu et al., 1963; Yang and Cheng, 1987; Zhang et al., 1994; Li, 1998). Therefore, the following diagnostic characteristics of *P. echinogaster* are proposed: dorsal fin VIII-XI-43–51, anal fin V-VIII-43–49, pectoral fin 22–27, caudal fin 19–22; transverse occipital

Table 3. Comparative counts of *P. echinogaster* in different studies

		Dorsal fin	Pectoral fin	Anal fin	Caudal fin	Gill rakers	Vertebrae
P. echinogaster	this study	VIII-XI-43–51	22–27	V-VIII-43–49	19–22	15–20	39–41
	Liu et al. (2013)	IX-XI-44–49	22–24	VI-VII-42–48	20–22	16–21	40–41
P. argenteus	Li et al. (2013)	VII-VIII-39–43	21–29	V-VI-35–41	26–28	10–12	37–38
	Liu et al. (2013)	IX-XI-44–48	22–24	VI-VII-43–47	20–22	16–19	40

canal on top of head moderately small, wavy ridges not reaching upper origin of pectoral fin; ventral branch of lateral line canal spare, shorter than dorsal branch of lateral line canal; gill rakers sparse, slender (pointed), 3–4+12–16=15–20; vertebrae 39–41. Our analysis indicates that *P. echinogaster* and *P. argenteus* can be clearly discriminated by the following diagnostic characteristics: (1) ventral branch of lateral line canal shorter than dorsal branch in *P. echinogaster* vs ventral branch of lateral line canal longer than dorsal branch in *P. argenteus*; (2) greater number of gill rakers on the first branchial arch in *P. echinogaster* (15–20 vs 10–12); (3) greater number of vertebrae in *P. echinogaster* (39–41 vs 37–38).

DNA barcoding (COI) is considered an effective and reliable method in the initial discrimination and identification of species (Hebert et al., 2003a). From the NJ tree, we determined that some COI sequences of *P. echinogaster* on GeneBank were incorrectly submitted as *P. argenteus*. Therefore, caution must be taken when employing GenBank data given the presence of such errors in the current release of GenBank. Based on the COI fragment analysis, the mean distance within Group 1 or Group 2 was below the species boundary (<2%) in the intraspecific distance range. These results suggests that all individuals within Group 1 belong to the same species. The genetic distance between Group 1 and Group 2 was 12.6%, which far exceeds the species threshold, this funding further indicates that Group 1 is conspecific with *P. echinogaster* and different from *P. argenteus*. These results are consistent with the phylogenetic relationships of the NJ tree as well as the variable site analyses. It has long been recognized that DNA sequence diversity, whether assessed directly or indirectly through protein analysis, can be used to discriminate among species. The number of amino acid mutations is significantly higher between the two groups than within each group. Some divergences occur at the genetic distance and amino acid levels that indicate that Groups 1 and 2 are different valid species. All lines of evidence suggest that the sequences of Group 1 would be regarded as DNA barcoding of *P. echinogaster*, whereas *Pampus* sp. (FJ652426 and FJ652427) are in fact *P. echinogaster*.

Based on a combination of sampling data and information from GenBank, we suggest that *P. echinogaster* is found in the Nel'ma Bight and northwestern coast of Sakhalin (Dolganov et al., 2007), Japan (Yamada et al., 2009), the Korean Peninsula (Oh et al., 2009), Bohai Sea (Nakabo, 2002; Yamada et al., 2009), Yellow Sea (Nakabo, 2002; Yamada et al., 2009), East China Sea (Nakabo, 2002; Yamada et al., 2009), and the northern waters of the South China Sea (Zhang and Hanner, 2012). In addition, we collected some specimen of this species from Zhuhai in 2016 that were not included in the present study.

The accurate and unambiguous identification of fish taxa is important for enabling the detection of retail substitutions of species, the management of fisheries for long-term sustainability, and the improvement of ecosystem research and conservation efforts. The resolution of cases of this nature will require careful morphological analysis by expert taxonomists before any final recommendations can be made (Ward et al., 2005). Mitochondrial sequence divergences are strongly linked to the process of speciation, and DNA barcoding and morphological analysis should be performed in a complementary manner. We hope that our results will provide more explicit species taxonomy and prevent the numerous misidentifications and erroneous distributional records within *Pampus*.

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