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Validity of *Pampus liuorum* Liu & Li, 2013, Revealed by the DNA Barcoding of *Pampus* Fishes (Perciformes, Stromateidae)

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Abstract: The genus *Pampus* contains seven valid species, which are commercially important fishery species in the Indo-Pacific area. Due to their highly similar external morphologies, *Pampus liuorum* has been proposed as a synonym of *Pampus cinereus*. In this study, partial sequences of COI (582 bp) and Cyt*b* (1077 bp) were presented as potential DNA barcodes of six valid *Pampus* species and the controversial species *P. liuorum*. A species delimitation of the seven *Pampus* species was performed to verify their validities. Explicit COI barcoding gaps were found in all assessed species, except for *P. liuorum* and *P. cinereus*, which resulted from their smaller interspecific K2P distance (0.0034–0.0069). A Cyt*b* barcoding gap (0.0200) of the two species was revealed, with a K2P distance ranging from 0.0237 to 0.0277. The longer Cyt*b* fragment is thus a more suitable DNA barcode for the genus *Pampus*. In the genetic tree, using concatenated Cyt*b* and COI sequences, the seven species reciprocally formed well-supported clades. Species delimitations with ABGD, GMYC, and bPTP models identified seven operational taxonomic units, which were congruent with the seven morphological species. Therefore, all of the seven analyzed species, including *P. liuorum*, should be kept as valid species.

Keywords: *Pampus liuorum* Liu & Li, 2013; DNA barcoding; species delimitation; systematics; Indo-West Pacific

Citation: Wei, J.; Wu, R.; Xiao, Y.; Zhang, H.; Jawad, L.A.; Wang, Y.; Liu, J.; Al-Mukhtar, M.A. Validity of *Pampus liuorum* Liu & Li, 2013, Revealed by the DNA Barcoding of *Pampus* Fishes (Perciformes, Stromateidae). *Diversity* **2021**, *13*, 618. https://doi.org/10.3390/d13120618

Academic Editor: Manuel Elias-Gutierrez

Received: 28 September 2021
Accepted: 23 November 2021
Published: 25 November 2021

1. Introduction

Pomfrets, species of genus *Pampus* Bonaparte, 1834, family Stromateidae Rafinesque, 1810, are pelagic marine fishes widely distributed along the coast of the Indo-West Pacific region. Seven valid species of genus *Pampus* have been recognized, namely, *Pampus argenteus* (Euphrasen, 1788), *P. candidus* (Cuvier, 1829), *Pampus chinensis* (Euphrasen, 1788), *Pampus cinereus* (Bloch, 1795), *Pampus minor* Liu & Li, 1998, *Pampus nozawae* (Ishikawa, 1904), and *Pampus punctatissimus* (Temminck & Schlegel, 1845) [1–9]. They contribute high commercial values to fisheries of the countries along the coast of the Indo-West Pacific region. In 2016, fishery harvests of pomfret in China reached over three million tons [10].

The taxonomy of the genus *Pampus* has long been confused by their highly similar external morphologies. *Pampus argenteus* might be the most confusing name in the genus *Pampus*. Its holotype is not available in its original description, while the vague original morphological description was found to be applicable to multiple known pomfret species [3]. Twelve available names were assigned as junior synonyms of *P. argenteus*,

including *P. minor*, *P. cinereus*, *P. candidus*, and *P. punctatissimus*, which have been recognized or resurrected as valid species [1,4,5,9]. Liu et al. [11] presented a morphological comparison of *P. argenteus*, *P. cinereus*, *P. chinensis*, *P. minor*, and *P. punctatissimus*, which indicated that the five species differed from each other in numerous external and skeletal characters, e.g., skull, gill rakers, and sensory canal systems on the head and lateral lines. Liu et al. [3], based on the original description and type locality of *P. argenteus*, redescribed the species and designated its neotype, which set up a reference for verifying validities of its junior synonyms. Simultaneously, the neotype of *P. cinereus* was assigned and described by Liu et al. [6] as a substitution of its lost holotype. Liu and Li [2] described a novel species, *Pampus liuorum* Liu & Li, 2013, based on its distinct morphology compared with six known pomfret species. However, the phylogenetic tree by Yin et al. [7], inferred from numerous nuclear gene loci, indicated that the specimens of *P. cinereus* and *P. liuorum* formed a mixing clade, refusing monophyly of the two species. *Pampus liuorum* is thus suspected to be a junior synonym of *P. cinereus* [7], and its monophyly and exclusiveness await further verification. Li et al. [12] proposed the resurrection of *P. echinogaster* from *P. argenteus* because of their distinct cytochrome *c* oxidase subunit I (COI) gene sequences. However, a morphological comparison indicated that *P. echinogaster sensu* Li et al. [12] is similar to the neotype of *P. argenteus* designated in Liu et al. [3], and thus could be a misidentification. *Pampus nozawae* used to be considered as a junior synonym of *P. cinereus* [6]. Its validity was recently proposed based on its distinct axial skeletal morphology comparing to its congeners [8], although a redescription and neotype designation of this species are currently unavailable. Therefore, the validities of *P. nozawae* and *P. echinogaster* are still uncertain. Radhakrishnan et al. [9] resurrected *P. candidus* based on its distinct morphological and genetic characteristics compared to *P. argenteus*, *P. cinereus*, and *P. liuorum*.

DNA barcoding, the idea of using short segments of genes to enable the precise identification of species, was proposed as an alternative way to clarify the species and genetic diversity of the genus *Pampus* [3,13,14]. Guo et al. [13] carried out preliminarily explorations on the genetic diversity of the genus *Pampus* using partial sequences of 16S ribosomal RNA (16S rRNA) and COI genes, and confirmed that *P. minor* was genetically distinct from its congeners. Cui et al. [14], using mitogenomic data, identified five species among specimens collected from the coast of China, i.e., *P. minor*, *P. punctatissimus*, *P. chinensis*, *P. cinereus*, and *Pampus* sp. (possibly *P. argenteus* or *P. echinogaster*). Li et al. [15] reported a new species, *Pampus* sp. nov., claiming its mitogenome to be different from its congeners. Radhakrishnan et al. [16] reported two new species, *Pampus* sp1. and *Pampus* sp2., from the Indian Ocean. Li et al. [17] presented an integrative comparison of morphological and genetic differences in seven *Pampus* species from the Indo-Pacific region. Neighbor-joining trees inferred from COI sequences suggested that *Pampus* sp1. and *Pampus* sp2. *sensu* (Radhakrishnan et al. [16]) are identical to *P. argenteus* and *Pampus* sp. nov. *sensu* (Li et al. [15]), respectively [17]. Despite the huge efforts, the misidentifications and mislabelings of the pomfret species frequently occur, especially on NCBI (National Center for Biotechnology Information) GenBank, which could hinder the application of DNA barcoding for *Pampus* species identification [7,17].

To establish reliable references for pomfret species identification, partial COI and cytochrome *b* gene (Cyt*b*) sequences of seven pomfret species are presented in this study as potential DNA barcodes. To verify the validity of the pomfret species, we performed phylogenetic inference and species delimitation with well-identified *Pampus* specimens collected from the Indo-Pacific region, including type specimens of *P. argenteus* and *P. liuorum* deposited in the Museum of Marine Biology, Institute of Oceanology, Chinese Academy of Sciences (IOCAS).

2. Materials and Methods

2.1. Sampling and Species Identification

In this study, seven pomfret species (74 specimens) were assessed (Figure 1): *Pampus argenteus*, *P. candidus*, *P. chinensis*, *P. cinereus*, *P. minor*, *P. liuorum*, and *P. punctatissimus*.

Due to a lack of specimens, *Pampus nozawae* was not included in this study. Six of the assessed *Pampus* species, including a total of seventy specimens, were collected from nine localities along the coast of China from August 2009 to January 2014 using commercial fishing trawl boats or gillnet fishing. Two paratypes of *P. liuorum* (i.e., IOCAS20120541 and 0542) were derived from Liu and Li [2], where the species was first described. Three specimens of *P. argenteus* (i.e., IOCAS120413, 0423, 0435) were derived from Liu et al. [6], where *P. argenteus* was redescribed. All specimens were carefully identified based on the type of specimen and our previous work on *Pampus* taxonomy [2–6]. Four specimens of *P. candidus* were collected from coastal Iraq in the northern Indian Ocean and identified based on morphological descriptions and the Cyt*b* sequences of Radhakrishnan et al. [9]. Muscle tissues of the specimens were taken and preserved in 95% ethanol for further experiments. All voucher specimens of the barcodes were deposited at the Museum of Marine Biology, IOCAS, Qingdao, China. Sequences of *Peprilus medius* (COI, AB205449; Cyt*b*, AB205471) from Doiuhi and Nakabo [18] were obtained from NCBI GenBank and selected as an outgroup for molecular analyses.

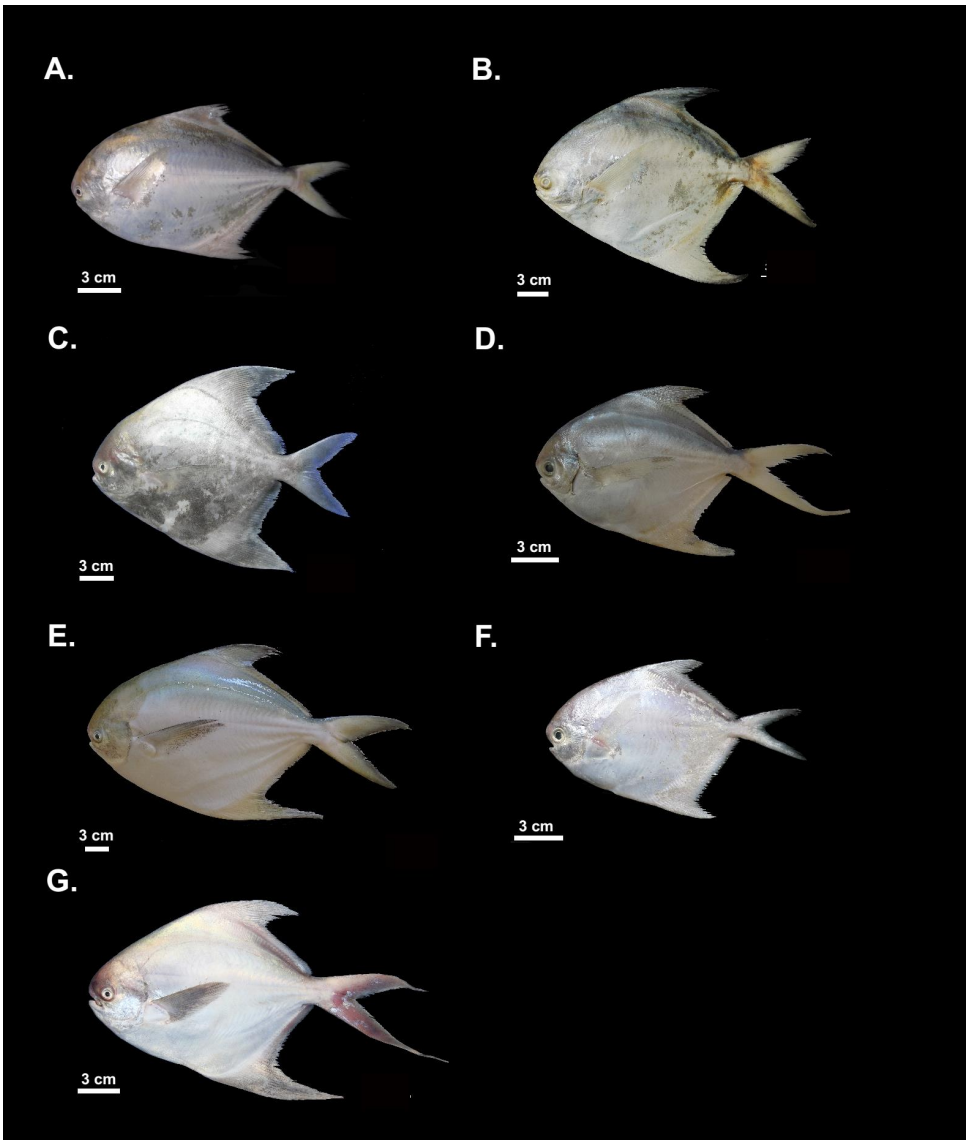

Figure 1. Photographs of the seven studied *Pampus* species of this study. (**A**) *P. argenteus* (PA-IOCAS120435); (**B**) *P. candidus* (PCA-2015004); (**C**) *P. chinensis* (PCH-201006003); (**D**) *P. cinereus* (PCI-20120520); (**E**) *P. liuorum* (PL-IOCAS120542); (**F**) *P. minor* (PM-2012504); (**G**) *P. punctatissimus* (PP-2013129).

2.2. DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from muscle tissues, following the protocol of Sambrook et al. [19]. The COI barcode sequence was amplified by two pairs of fish-specific primers (FishF1 and FishR1; FishF2 and FishR2) [20]. Based on mitochondrial genome sequences of *Pampus* in Cui et al. [14], a new primer (Thr20-Pam) was designed, and three primers reported by Doiuhi and Nakabo [18] were modified to form three pairs of primers for Cyt*b* sequence amplification of the genus *Pampus*. The primer names and sequences are as follows: one forward primer: L14724-Pam (5′-GACTTGAAAAACCATCGTTG-3′); three reverse primers: Thr20-Pam (5′-GTTTACAAGACCGGCGCTCT-3′), H15915-Pam (5′-TTCCGACGTCCGGTTTACAAGAC-3′), and H15973-Strdei (5′-TTGGGAGYYRGTGGTAG-GAGTT-3′). Polymerase chain reactions (PCR) were performed in a 50 µL volume with 50 ng template DNA, 5 µL of 10 × reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mixture, 0.2 µM of each primer, and 2.5 U *Taq* DNA polymerase (Transgen Biotech Co., Ltd., Beijing, China). PCR cycles were conducted on a Veriti^{TM} 96-Well Thermal Cycler (Applied Biosystems, USA) under the following protocol: initial denaturation for 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 50–52 °C, 45 s at 72 °C, and a final 10 min extension at 72 °C. PCR amplification without the addition of the template DNA was used as a negative control reaction to ensure no cross-contamination during the experiments. PCR products were separated on 1.2% agarose gel, and then sent to Sangon Biotech Co., Ltd. (Shanghai, China) for bidirectional DNA sequencing with the corresponding forward and reverse primers in PCR reactions, using the ABI Prism 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Phylogenetic Inference and Barcoding Gaps

The raw sequences were first assembled in EditSeq V7.1.0 (Lasergene, DNASTAR, Madison, WI, USA), and only high-quality bases with clear signals were retained for analyses. Sequence alignment was carried out in MegAlign V7.1.0 (Lasergene, DNASTAR) using the ClustalW algorithm with default settings. The sequences were trimmed to obtain uniform lengths for subsequent analyses. The COI and Cyt*b* sequences were deposited in NCBI GenBank. Sampling information, specimen photos (whenever available), and corresponding COI and Cyt*b* sequences of the specimens were also archived on the Barcoding of Life Database (BOLD) under a public project coded by IOCAS (https://www.boldsystems.org/index.php/Public_SearchTerms?query=IOCAS, access on 1 November 2021). Sampling information, voucher specimen numbers (Museum ID of BOLD), and NCBI GenBank accession numbers of COI and Cyt*b* of the specimens are summarized in Table 1. Sequence variation indices of COI and Cyt*b* sequences among *Pampus* species, including base composition and number of polymorphic sites, parsimony informative sites, and indels, were calculated using DnaSP v6 [21]. COI and Cyt*b* sequences of the specimens were concatenated to form another dataset for tree inferences and species delimitations.

Table 1. Sampling data, BOLD sample IDs, and GenBank accession numbers of the *Pampus* species used in this study. The"✓" sign indicates that the specimen photo is available on BOLD.

Species	Sampling Date	Sampling Location (Number of Specimens)	BOLD Specimen Voucher		GenBank Accession Number	
			Museum ID	Photo Reference	COI	Cyt*b*
Pampus argenteus	April 2012	Zhuhai, Guangdong, China (3)	PA-IOCAS120413	✓	MK300954	MK301024
			PA-IOCAS120423	✓	MK300957	MK301027
			PA-IOCAS120435	✓	MK300958	MK301028

Table 1. *Cont.*

Species	Sampling Date	Sampling Location (Number of Specimens)	BOLD Specimen Voucher		GenBank Accession Number	
			Museum ID	Photo Reference	COI	Cyt*b*
	April–May 2012	Shenzhen, Guangdong, China (6)	PA-20120418	✓	MK300955	MK301025
			PA-20120419	✓	MK300956	MK301026
			PA-20120443	✓	MK300959	MK301029
			PA-20120444	✓	MK300960	MK301030
			PA-20120445	✓	MK300961	MK301031
			PA-20120447	✓	MK300962	MK301032
	May 2012	Zhanjiang, Guangdong, China (3)	PA-20120531	✓	MK300963	MK301033
			PA-20120532	✓	MK300964	MK301034
			PA-20120533	✓	MK300965	MK301035
	January 2014	Weihai, Shandong, China (4)	PA-201401001		MK300988	MK301058
			PA-201401002		MK300989	MK301059
			PA-201401003		MK300990	MK301060
			PA-201401004		MK300991	MK301061
	April 2012	Qingdao, Shandong, China (7)	PA-20120401	✓	MK300981	MK301051
			PA-20120402	✓	MK300982	MK301052
			PA-20120403	✓	MK300983	MK301053
			PA-20120404	✓	MK300984	MK301054
			PA-20120405		MK300985	MK301055
			PA-20120406		MK300986	MK301056
			PA-20120409		MK300987	MK301057
	May 2012	Zhoushan, Zhejiang, China (3)	PA-EZ2012003	✓	MK300992	MK301062
			PA-EZ2012004	✓	MK300993	MK301063
			PA-EZ2012005	✓	MK300994	MK301064
Pampus candidus	January 2015	Iraq (4)	PCA-2015004	✓	MZ604279	MZ604560
			PCA-2015005		MZ604280	MZ604561
			PCA-2015006		MZ604281	MZ604562
			PCA-2015007		MZ604282	MZ604563
Pampus chinensis	August 2009	Xiamen, Fujian, China (1)	PCH-200908009	✓	MK300966	MK301036
	May 2010	Zhuhai, Guangdong, China (5)	PCH-2010050025	✓	MK301037	MK300967
			PCH-2010050027	✓	MK301038	MK300968
			PCH-201006001	✓	MK301039	MK300969
			PCH-201006002	✓	MK301040	MK300970
			PCH-201006003	✓	MK301041	MK300971
Pampus cinereus	April–May 2012	Shenzhen, Guangdong, China (3)	PCI-20120457	✓	MK300972	MK301042
			PCI-20120459	✓	MK300973	MK301043
			PCI-20120460	✓	MK300974	MK301044
	April–May 2012	Zhuhai, Guangdong, China (3)	PCI-20120464	✓	MK300975	MK301045
			PCI-20120465	✓	MK300976	MK301046
			PCI-20120481	✓	MK300977	MK301047
	May 2012	Zhanjiang, Guangdong, China (3)	PCI-20120520	✓	MK300978	MK301048
			PCI-20120521	✓	MK300979	MK301049
			PCI-20120522	✓	MK300980	MK301050
Pampus liuorum	May 2012	Zhuhai, Guangdong, China (2)	PL-IOCAS120541	✓	MK300995	MK301065
			PL-IOCAS120542	✓	MK300996	MK301066

Table 1. *Cont.*

Species	Sampling Date	Sampling Location (Number of Specimens)	BOLD Specimen Voucher		GenBank Accession Number	
			Museum ID	Photo Reference	COI	Cyt*b*
	July–August 2013	Dongshan, Fujian, China (9)	PL-20130726061		MK300997	MK301067
			PL-20130726062		MK300998	MK301068
			PL-20130726063		MK300999	MK301069
			PL-20130726064		MK301000	MK301070
			PL-20130726065		MK301001	MK301071
			PL-20130810031		MK301002	MK301072
			PL-20130726066		MK301003	MK301073
			PL-20130810029		MK301004	MK301074
			PL-20130810030		MK301005	MK301075
Pampus minor	October 2013	Zhoushan, Zhejiang, China (1)	PM-2013159		MK301013	MK301083
	April 2012	Shenzhen, Guangdong, China (1)	PM-20120430	✓	MK301006	MK301076
	May 2010	Zhuhai, Guangdong, China (2)	PM-S20-098		MK301014	MK301084
			PM-S20-102	✓	MK301015	MK301085
	May 2012	Zhanjiang, Guangdong, China (3)	PM-20120503	✓	MK301007	MK301077
			PM-20120504	✓	MK301008	MK301078
			PM-20120513	✓	MK301009	MK301079
	April 2013	Beihai, Guangxi, China (3)	PM-2013065		MK301010	MK301080
			PM-2013066		MK301011	MK301081
			PM-2013067		MK301012	MK301082
Pampus punctatissimus	June 2013	Zhoushan, Zhejiang, China (2)	PP-20130618	✓	MK301017	MK301087
			PP-20130619	✓	MK301018	MK301088
	October 2013	Xiamen, Fujian, China (5)	PP-2013129	✓	MK301019	MK301089
			PP-2013138		MK301020	MK301090
			PP-2013139		MK301021	MK301091
			PP-2013146		MK301022	MK301092
			PP-2013154		MK301023	MK301093
	April 2012	Zhuhai, Guangdong, China (1)	PP-20120427	✓	MK301016	MK301086

Due to more genetic distance references for Kimura's two-parameter model (K2P) [22], we calculated pairwise K2P distances to estimate barcoding gaps of each species. K2P distances among and within the identified *Pampus* species, namely, interspecific and intraspecific K2P distances, were calculated in MEGA7 using the COI and Cyt*b* datasets [23]. Interspecific and intraspecific K2P distances of each species were visualized using boxplots in OriginPro 2020 (OriginLab ©, Northampton, MA, USA). The barcoding gap for each species was then calculated as the difference between the minimum interspecific distance and the maximum intraspecific distance [24,25].

Three datasets were used for phylogenetic inference, i.e., the COI dataset, the Cyt*b* dataset, and concatenated datasets of the two genes. Specially, COI and Cyt*b* sequences were treated as two partitions in the concatenated dataset. Best-fit models available in IQtrees v 1.6.12 [26] and MrBayes v 3.2 [27] were selected in jModelTest 2 [28] using the Akaike information criterion [29]. The best fit models for COI and Cyt*b* were HKY + G + I [30] and GTR + G [31]. Maximum likelihood trees were inferred in IQtrees v1.6.12 [26], with 1000 bootstrap replicates to estimate the bootstrap values (BSs) of nodes. For BI trees, two independent Markov chain Monte Carlo (MCMC) runs were performed in MrBayes v3.2, with four chains for 500,000 generations, sampling every 100 generations and discarding the first 25% of samples as burn-ins [27]. Sufficient convergence of the

runs was evaluated with summary statistics in MrBayes v3.2 (effective sampling size > 200, potential scale reduction factors≈1). All phylogenetic trees were rooted by the outgroup *Peprilus medius*.

2.4. Species Delimitation

Species delimitation was performed with the concatenated dataset of COI and Cyt*b* using a distance-based method, i.e., automatic barcode gap discovery (ABGD) [32], and two tree-based methods, i.e., the single threshold Bayesian Poisson tree processes (bPTP) model and the generalized mixed Yule-coalescent (GMYC) model [33–35]. The ABGD attempts to identify the barcoding gap as the first significant gap in pairwise distances among a given sequence dataset and uses the detected gap to partition the data [32]. The ABGD was performed on an online ABGD interface of Muséum National d'Histoire Naturelle, France (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html, access on 1 November 2021), scanning a range of prior intraspecific divergence values from 0.1% to 10% with 50 search steps and default settings, although applying K2P distances [22] instead of Jukes-Cantor distances [36].

The single-threshold GMYC identifies speciation events by detecting apparent branching rate increases at the transition of interspecific diversification to population-level coalescence. The GMYC model requires inputs of ultrametric trees; therefore, the ultrametric tree of the concatenated dataset was generated using BEAST2 v 2.5.1 [37], applying prior best-fit models of the two genes, the lognormal relax clock model, and constant population size coalescent tree. Specially, the root node height was constrained to an arbitrary age of 1. Two parallel MCMC runs were performed for 50,000,000 generations, with sampling trees and parameters every 1000 generations. Logfiles were combined in LogCombiner v. 2.5.1 and subsequently analyzed with Tracer v. 1.7 of the BEAST2 package. Sufficient convergence of the two runs was checked by the convergence of parameter values, and ESS should be greater than 200. Trees were summarized with TreeAnnotator v. 2.5.1 and visualized in FigTree v 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/, access on 1 November 2021). The Newick ultrametric tree was uploaded to the Exelixis Lab web interface for GMYC modeling (https://species.h-its.org/gmyc/, access on 1 September 2021).

bPTP modeling was also performed on the Exelixis Lab web interface (https://species.h-its.org/, access on 1 September 2021). The bPTP model is an updated version of the original maximum likelihood PTP model, with both the implementation of maximum likelihood searches and Bayesian analyses. Similar to GMYC modeling, the bPTP model delimitates speciation events based on a shift in the number of substitutions between internal nodes instead of time [38,39]. It requires a distance-based phylogram instead of a time-based ultrametric tree [40], and thus might eliminate an error-prone step of divergence time inference that potentially affects the previous method. The Newick tree file for bPTP modeling was generated in MrBayes v 3.2 using the concatenated dataset of COI and Cyt*b*. The settings for MrBayes v 3.2 were the same as those described in Section 2.3.

3. Results

3.1. Sequence Variation Indices and Barcoding Gaps of COI and Cytb

For COI and Cyt*b*, 582 and 1077 bp sequences were retrieved from each specimen collected in this study, respectively; no indel was found in either dataset. The two datasets were concatenated and formed a 1659 bp dataset. Average base compositions (A:G:C:T) of the COI and Cyt*b* datasets were 0.248:0.175:0.246:0.330 and 0.266:0.131:0.292:0.311. Among the seven assessed *Pampus* species, the 582 bp COI dataset contained 167 polymorphic sites, including 132 parsimony informative sites. The 1077 bp Cyt*b* dataset contained 361 polymorphic sites, including 284 parsimony informative sites. Pairwise COI K2P distances among the seven *Pampus* species (i.e., interspecific distances) ranged from 0.0034 to 0.1823, and pairwise COI K2P distances within each species (i.e., intraspecific distances) ranged from 0.0000 to 0.0052 (Table 2). COI barcoding gaps have been well identified in five species, i.e., *P. argenteus*, *P. candidus*, *P. chinensis*, *P. minor*, and *P. punctatissimus*

(Figure 2), with their values ranging from 0.0104 to 0.1221 (Table 2). In contrast, the COI barcoding gaps of *P. cinereus* and *P. liuorum* were found to be very small (0.0017 and 0.0000, respectively; Figure 2 and Table 2), which resulted from smaller pairwise K2P distances comparing sequences of *P. cinereus* and *P. liuorum* (0.0034–0.0069). For the Cyt*b* dataset, interspecific K2P distances among the seven species ranged from 0.0237 to 0.1850, whereas intraspecific K2P distances ranged from 0.0000 to 0.0065. The Cyt*b* barcoding gaps have been well identified in all seven species, with the values being 0.0200–0.1452. The smallest Cyt*b* barcoding gap (0.0200) has been observed in *P. cinereus* and *P. liuorum*.

Table 2. Interspecific and intraspecific K2P distances of the seven analyzed *Pampus* species.

Species	COI			Cyt*b*		
	Interspecific	Intraspecific	Barcoding Gap	Interspecific	Intraspecific	Barcoding Gap
Pampus argenteus	0.1273–0.1572	0.0000–0.0052	0.1221	0.1508–0.1809	0.0000–0.0056	0.1452
Pampus candidus	0.0139–0.1556	0.0000–0.0034	0.0105	0.0355–0.1849	0.0009–0.0065	0.0290
Pampus chinensis	0.0580–0.1823	0.0000–0.0034	0.0545	0.0555–0.1790	0.0000–0.0028	0.0527
Pampus cinereus	0.0034–0.1799	0.0000–0.0017	0.0017	0.0237–0.1850	0.0000–0.0037	0.0200
Pampus liuorum	0.0034–0.1572	0.0000–0.0034	0.0000	0.0237–0.1811	0.0000–0.0037	0.0200
Pampus minor	0.1318–0.1572	0.0000–0.0034	0.1283	0.1698–0.1850	0.0000–0.0047	0.1651
Pampus punctatissimus	0.0580–0.1427	0.0000–0.0034	0.0545	0.0555–0.1777	0.0000–0.0056	0.0499
Overall	0.0034–0.1823	0.0000–0.0052	−0.0018	0.0237–0.1850	0.0000–0.0065	0.0172

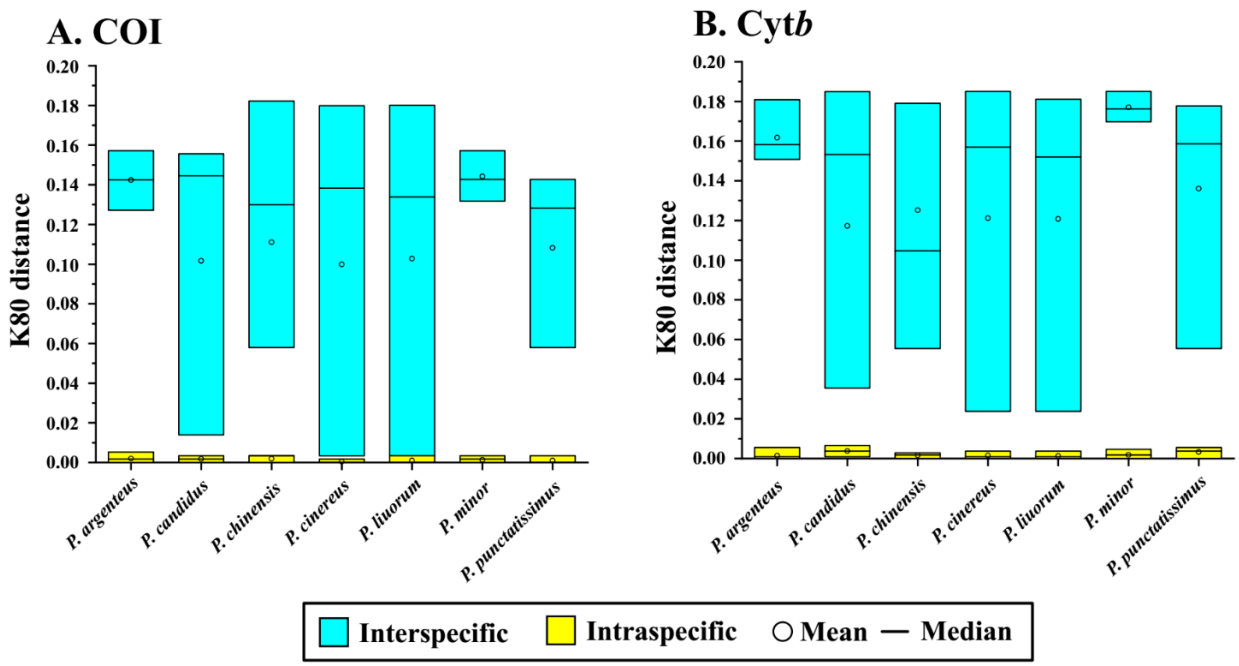

Figure 2. Boxplot showing the COI (**A**) and Cyt*b* (**B**) barcoding gaps of the studied species in genus *Pampus*. Pairwise interspecific and intraspecific K2P distances of each species are annotated with blue and yellow. Mean and median values of the interspecific and intraspecific distances are indicated with circles and lines, respectively.

3.2. Phylogenetic Inference

Maximum likelihood and BI trees retrieved from COI and Cyt*b* datasets singly recovered well-supported clades, corresponding to the morphologically identified species. In the COI tree (Figure 3A), five well-supported clades (BS = 81–100; posterior probabilities, PP = 1) can be identified, which are, based on their morphological identification, *P. argenteus*, *P. minor*, *P. chinensis*, *P. punctatissimus*, and a mix clade of *P. liuorum*, *P. cinereus*, and *P. candidus*. The COI sequences of *P. liuorum*, *P. cinereus*, and *P. candidus* do not form monophyla reciprocally. Instead, sequences of the three species form a single well-

supported (BS = 81; PP = 1) clade, with the COI sequences of *P. candidus* and *P. cinereus* being two monophyla nested within it (Figure 3A). For Cyt*b* trees (Figure 3B), the sequences of the seven morphological species, i.e., *P. argenteus*, *P. minor*, *P. chinensis*, *P. punctatissimus*, *P. liuorum*, *P. cinereus*, and *P. candidus*, form monophyla reciprocally, which are well supported by BS values of 93–100, and a PP value of 1 (Figure 3B). *Pampus liuorum* has been resolved as a sister species of *P. cinereus* (BS = 74; PP = 0.84), whereas *P. candidus* is closely linked to the two species (BS = 100; PP = 1, Figure 3B). A sister relationship between *P. argenteus* and *P. minor* is indicated in the Cyt*b* tree, although it is supported by a relatively low PP value (PP = 0.87, Figure 3B).

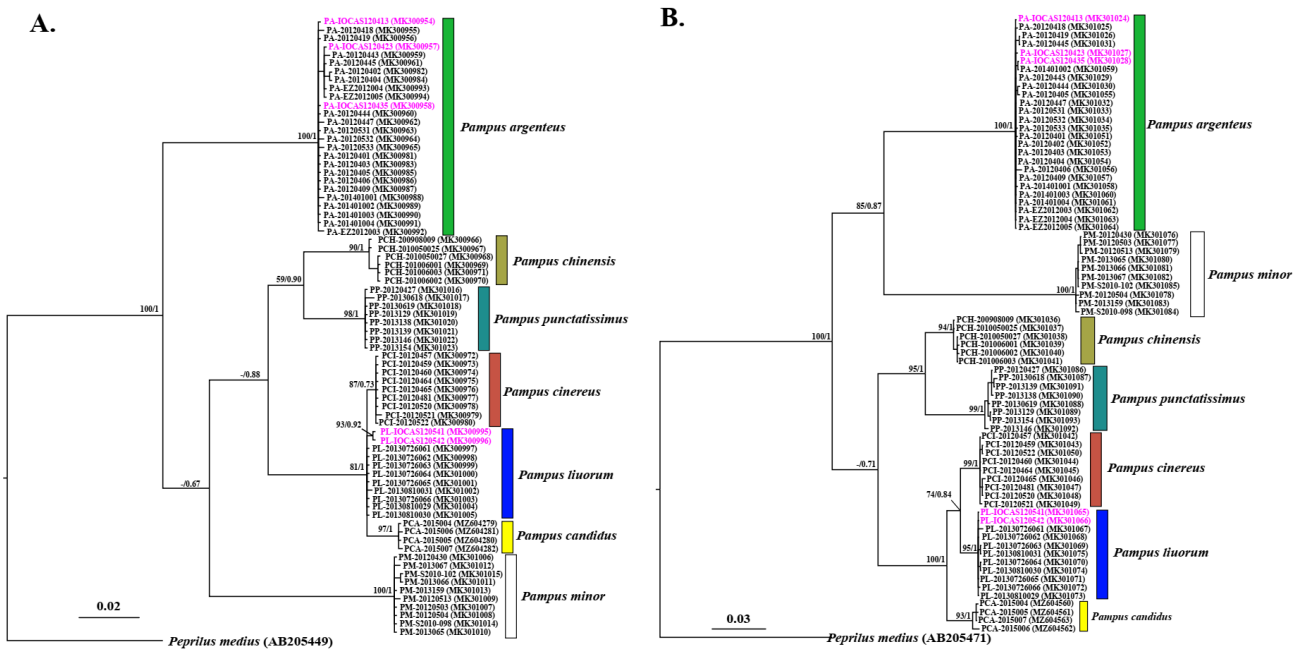

Figure 3. Maximum likelihood tree of the *Pampus* species inferred from COI (**A**) and Cyt*b* (**B**) datasets. Bootstrap values (before slash) and posterior probabilities (after slash) are shown on each node; "-" on the node indicates that the node was not included in the maximum likelihood or Bayesian analyses. Specimens in purple are specimens derived from Liu and Li [2] and Liu et al. [3].

Similar to the Cyt*b* trees, phylogenetic trees retrieved from concatenated datasets of COI and Cyt*b* well support the monophyly of all seven morphological species (BS = 98–100; PP = 1, Figure 4). The topology of the ML and BI trees is almost identical, except for the different relationships of *P. candidus*, *P. liuorum*, and *P. cinereus*. In the ML tree, *Pampus liuorum* is a sister to *P. cinereus* (BS = 60), whereas *P. candidus* is closely linked to the two species (Figure 4). In the BI tree, *Pampus candidus* is resolved as a sister species of *P. cinereus* (PP = 0.51). In both the ML and BI trees, *Pampus argenteus* is resolved as a sister of *P. minor* (BS = 78, PP = 0.64, Figure 4).

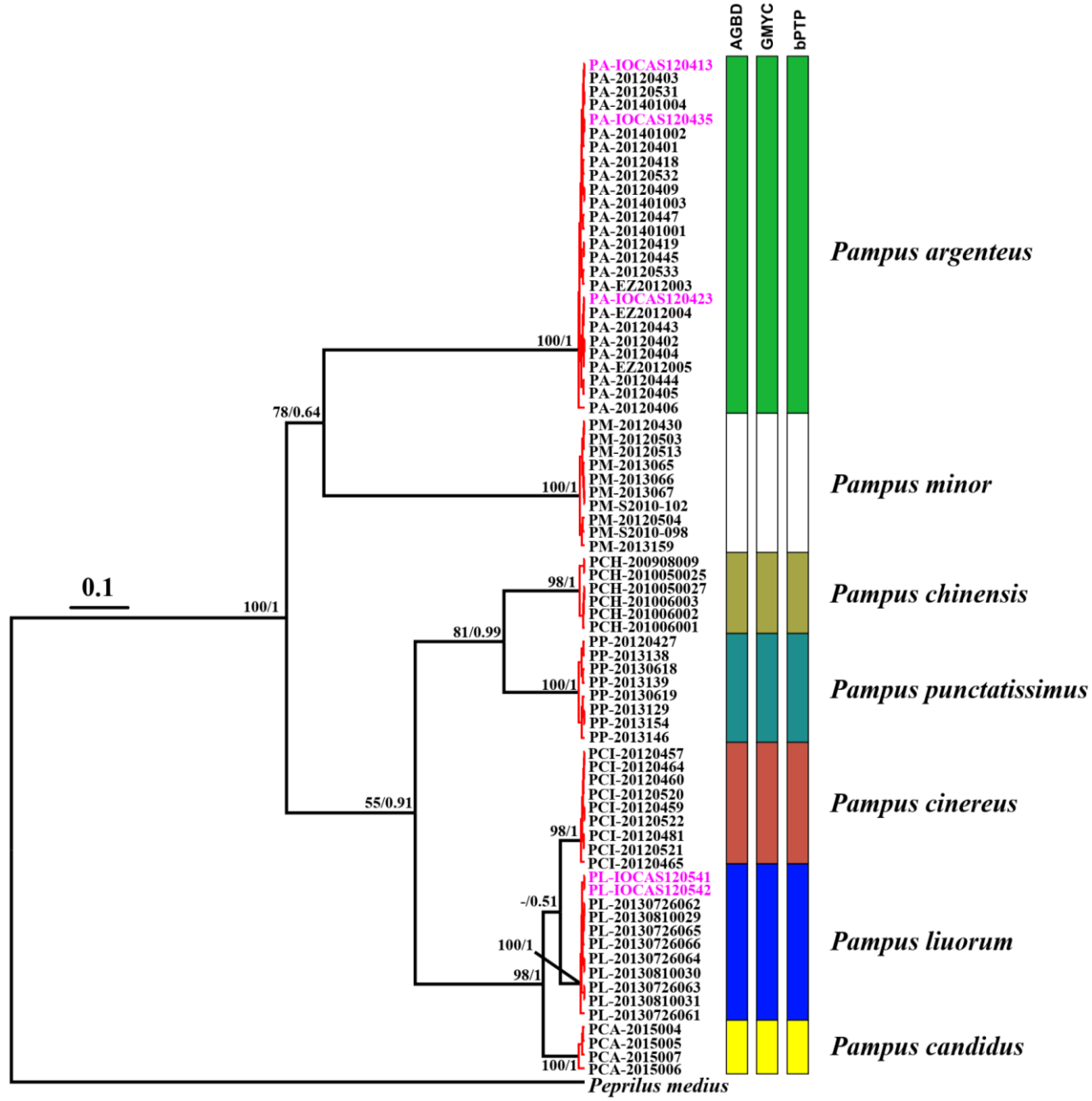

Figure 4. Maximum likelihood tree of the *Pampus* species inferred from concatenated dataset of COI and Cyt*b* sequences. Bootstrap values (before slash) and posterior probabilities (after slash) are shown on each node; "-" on the node indicates that the node was not included in the maximum likelihood or Bayesian analyses. Results of the two species delimitation methods, i.e., the GYMC and bPTP model, are shown on the left. Specimens in purple are specimens derived from Liu and Li [2] and Liu et al. [3].

3.3. Species Delimitation

Species delimitation with the ABGD, GMYC, and bPTP methods using the concatenated dataset consensually concluded seven operational taxonomic units (OTUs) among the analyzed *Pampus* specimens, which are congruent with the seven morphological species (Figure 4). The ABGD method indicated that the first detected significant barcoding gap was 0.0166. The number of OTUs was reduced from seven to five when applying a larger prior maximum intraspecific K2P distance, e.g., the next maximum intraspecific K2P distance value scanned by the ABGD, 0.0184, which suggested that seven putative species were delimitated with the first barcoding gap detected. The GMYC model delimited seven

OTUs as the maximum likelihood solution, which was also the only solution in the confidence interval. The likelihood ratio test of the GMYC model showed highly significant differences ($p < 0.001$) between the maximum likelihood (-Log $L_{GMYC\text{-}max}$ = 671.454) of the GMYC model and likelihood (-Log L_{Null} = 649.49) of the null model (i.e., assuming only one species among all analyzed specimens). The likelihood ratio test therefore refuted the null model and supported the alternative hypothesis, i.e., the seven species delimitation. The bPTP modeling detected the seven most supported partitions among all analyzed specimens. The delimitation support values for each morphological species are as follows: *P. argenteus*, 0.95; *P. minor*, 0.91; *P. chinensis*, 0.97; *P. punctatissimus*, 0.88; *P. candidus*, 0.82; *P. cinereus*, 0.96; and *P. liuorum*, 0.92.

4. Discussion

4.1. Pampus cinereus, Pampus liuorum, and Pampus candidus as Distinct Valid Species

Both phylogenetic inferences of COI and Cyt*b* implied a relatively closer evolutionary relationship of *P. candidus*, *P. liuorum*, and *P. cinereus*. The K2P distances between each of these three species (COI, 0.0034–0.0210; Cyt*b*, 0.0237–0.0277) were relatively small compared with those of other species pairs (COI, 0.0580–0.1572; Cyt*b*, 0.0555–0.1850), which might imply a close phylogenetic relationship and more recent origin of these three species. Phylogenetic trees retrieved from COI, Cyt*b*, and the concatenated dataset of the two genes congruently resolved the three species as a monophyletic group, well supported by BS values of 81–100 and PP values of 1 (Figures 3 and 4). A close relationship of *P. cinereus* and *P. candidus* was also supported by the phylogenetic tree in Radhakrishnan et al. [9]. However, our phylogenetic inference is based on only two mitochondrial gene fragments, which might account for the low support values in the trees and the inconsistency between the BI and ML trees (Figure 4). The phylogeny of the genus *Pampus* needs to be clarified with larger genetic datasets in the future.

Despite their close genetic relationships, the three species are clearly delineated as different species in the ABGD, GMYC, and bPTP models (Figure 4). Liu and Li [2] illustrated that *P. liuorum* could be distinguished from *P. cinereus* by the following characteristics: shorter pectoral fins [31.5–41.7% standard length (SL) vs. 42.0–47.2% SL]; more vertebrae (38 vs. 36); when alive, with golden bronze or yellowish blue color on its back (vs. *P. cinereus*, whole body silvery grey, anal fin and ventral side sometimes yellow). Although the total vertebral counts of *P. cinereus* and *P. liuorum* were claimed to be identical (37 vertebrae) in Jawad and Liu [8], the actual numbers of total vertebrae counted from their radiographs were 36 (*P. cinereus*, Figure 3C in Jawad and Liu [8]) and 38 (*P. liuorum*, Figure 1A in Jawad and Liu [8]), which agrees with the descriptions in Liu and Li [2]. The recently resurrected *P. candidus* possesses an intermediate number of total vertebrae (37 vertebrae) between *P. cinereus* (36 vertebrae) and *P. liuorum* (38 vertebrae) [9]. It could also be discriminated from *P. liuorum* by having fewer vertebrae (14 vs. 15) between the first pterygiophore of dorsal and anal fins [9]. Therefore, the total vertebral count is an exclusive and conservative characteristic in identifying the three species. Yin et al. [7] proposed a synonymy of *P. cinereus* and *P. liuorum*, because their phylogenetic analysis using numerous nuclear genes indicated a mixing clade of *P. cinereus* with *P. liuorum*. In fact, the mixing clade of *P. liuorum* and *P. cinereus* contains three well-supported clades (BS = 100), i.e., a clade of *P. cinereus*, a clade of *P. liuorum*, and a mixed clade formed of two "*P. cinereus*" and "*P. liuorum*" specimens. The genetic distances among the three clades (approximately 0.0056–0.0100) were similar to those between *P. chinensis* and *P. punctatissimus* (approximately 0.0073–0.0144, Figure 1 in Yin et al. [7]), implying that the three clades might contain three species. The total vertebral counts of *P. cinereus* (36–37) and *P. liuorum* (36–38) varied between the estimated specimens in Yin et al. [7], which was incongruent with those recorded in Liu and Li [2]. Yin et al.'s [7] conclusion on the synonymy of *P. cinereus* and *P. liuorum* might be based on misidentified specimens, and might therefore be incorrect. Our analyses indicate that the well-identified specimens of *P. liuorum*, including the paratypes of the species (i.e., IOCAS120541, 0542), are delineated as a single species, which

is clearly distinct from *P. cinereus* and *P. candidus* (Figure 4). It supports that *P. liuorum* described in Liu and Li [2] is a valid species. On this basis, the genus *Pampus* now contains eight recognized valid species: *Pampus argenteus* (Euphrasen, 1788), *P. candidus* (Cuvier, 1829), *Pampus chinensis* (Euphrasen, 1788), *Pampus cinereus* (Bloch, 1795), *Pampus liuorum* Liu & Li, 2013, *Pampus minor* Liu & Li, 1998, *Pampus nozawae* (Ishikawa, 1904), and *Pampus punctatissimus* (Temminck & Schlegel, 1845); however, *P. nozawae* needs further taxonomic revision in order to clarify its validity.

4.2. Species Delimitation and Validity of Pampus argenteus

Our phylogenetic trees and species delimitation analyses (ABGD, GMYC, and bPTP model) also support the validity of *P. argenteus* (Figure 4). The identity of *P. argenteus* used to be disputed because of its lack of holotype and the vague original morphological description upon its first publication, which could be applied to multiple known pomfret species [6,10,14]. To solve this taxonomic problem, Liu et al. [6] redescribed the species and designated its neotype—the neotype is assigned as the new name-bearing type for *P. argenteus*. Concurrently, Liu et al. [6] listed a series of non-type specimens identified as *P. argenteus*, which are alternative morphological references of *P. argenteus*. In our genetic analyses (Figures 3 and 4), three of these non-type specimens (i.e., IOCAS120413, IOCAS120423, and IOCAS120435) formed a well-supported clade with the other *P. argenteus* specimens, which were delineated as a single species in ABGD, GMYC and bPTP modeling (Figure 4). *Pampus argenteus* could be distinguished from its congeners by having a combination of the following characters: mouth subterminal (vs. mouth terminal, *P. chinensis* and *P. punctatissimus*); eyes small, with an eye diameter 24.6–27.1% of head length (vs. 27.3–36.4% of head length, *P. minor*); more vertebrae, a total vertebral count of 40 (vs. 32–38, other *Pampus* species); and dorsal and anal fins with short falcate lobes (vs. fins with long falcate lobes, *P. cinereus*, *P. liuorum*, *P. candidus*, and *P. punctatissimus*) [2,6,9,11]. *Pampus argenteus* redescribed in Liu et al. [6] is thus a valid species with exclusive morphological and genetic characteristics.

4.3. Verification of COI and Cytb as Potential DNA Barcodes for Pomfret Identification

In this study, both COI and Cyt*b* exhibited certain abilities to identify species of the genus *Pampus*, although the shorter fragment of COI failed to distinguish the closely related species *P. candidus*, *P. liuorum*, and *P. cinereus*. The anterior region of COI (~600 bp, amplified from universal primer pairs for fish, e.g., FishF1 and Fish R1; Fish F2 and Fish R2 [20]; VF1 and VR1 [41]) is a common DNA barcode for fish identification [42,43]. It has widely been applied in various areas, including fishery management [42,44,45] and the forensic investigation of smuggled fish products [46]. Barcoding gaps between intraspecific and interspecific genetic distance have frequently been reported in mitochondrial barcodes among a vast number of fish taxa, with the intraspecific genetic difference rarely exceeding 2% [47–49]. The 2% genetic difference in mitochondrial genes could thus be empirically accepted as a general boundary and standard for distinguishing interspecific and intraspecific divergence [42,50,51]. In our study, 582 bp of the common COI barcodes were obtained for *Pampus* species using the two primer pairs from Ward et al. [20]. Explicit barcoding gaps (Figure 2) were found in five of the analyzed species, i.e., *P. argenteus*, *P. candidus*, *P. chinensis*, *P. minor*, and *P. punctatissimus*. Nevertheless, our result showed no obvious COI barcoding gap for *P. cinereus* and *P. liuorum*, although there were only 2–4 bp differences among their COI sequences. The shorter traditional COI barcode (586 bp) could contain insufficient variant information to distinguish the two species. The fragments of Cyt*b* have recently been applied as alternative barcodes for fish identification [52,53]. Comparative analyses of COI, Cyt*b*, 16S rRNA, and 18S rRNA suggested that Cyt*b* possesses a higher level of sequence variation among fish species [53]. In this study, analyses on 1077 bp partial Cyt*b* sequences clearly verified barcoding gaps for all seven pomfret species, with the maximum intraspecific K2P distance and minimum interspecific K2P distance being 0.0065 and 0.0237, respectively (Figure 2 and Table 2). Phylogenetic inference using Cyt*b*

sequences supported the monophyly of each analyzed species (Figure 3B). This suggests that the longer fragments of Cyt*b* could provide more variant information than the traditional barcoding region of COI in identifying *Pampus* species. Therefore, adopting longer fragments of Cyt*b* as the DNA barcode could be a recommended strategy to ascertain the accurate identification of pomfret species.

5. Conclusions

In this study, we have evaluated partial sequences of the COI (582 bp) and Cyt*b* (1077 bp) of seven *Pampus* species as their potential DNA barcodes. Cyt*b* barcoding gaps have been identified in all assessed species, whereas COI barcoding gaps were not identified in *P. cinereus* and *P. liuorum*, which suggests that the longer fragment of Cyt*b* would be a more suitable barcode for the genus *Pampus*. Species delimitations have been performed with GMYC and bPTP models to assess the validities of the seven collected species. Both delimitation methods identified seven OTUs, which were congruent with the seven morphological species. Therefore, we proposed the seven analyzed species, including the controversial species *Pampus liuorum* Liu & Li, 2013, as valid species.

Author Contributions: Conceptualization, J.L. and R.W.; software, J.W. and R.W.; formal analysis, J.W., R.W. and H.Z.; data curation, J.W., R.W., J.L. and M.A.A.-M.; investigation, R.W., Y.X., L.A.J., J.L. and M.A.A.-M.; writing—original draft preparation, J.W. and R.W.; writing—review and editing, J.W., R.W., Y.X., L.A.J., Y.W., J.L. and M.A.A.-M.; visualization, J.W.; supervision, J.L. and Y.W.; funding acquisition, R.W., Y.W. and J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study is funded by National Natural Science Foundation of China (Nos. 31872195 and 31772869), Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDB42000000), and the Science and Technology Planning Project of Guangdong Province, China (No. 2017A030303077).

Institutional Review Board Statement: The experimental animal protocols in the present study have been reviewed and approved by the Animal Experimental Ethics Committee of Institute of Oceanology, Chinese Academy of China (approval number: 0928-2021). Experiment procedures were performed in accordance with the Provisions and Regulations for the National Experimental Animal Management Regulations (China, July 2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: All of the COI and Cyt*b* data used in this study have been deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov, access on 1 November 2021) with the accession numbers MK300954–MK301093, MZ604279–MZ604282, and MZ6042560–MZ6042563. The COI and Cyt*b* sequences have also been deposited in BOLD under project code IOCAS and the title of this study (https://www.boldsystems.org/index.php/Public_SearchTerms?query=IOCAS, access on 1 November 2021).

Acknowledgments: The authors are thankful to Chun-sheng Li of the Institute of Oceanology, Chinese Academy of Sciences, China, for sharing part of his *Pampus* collections and useful information on species identification with us.

Conflicts of Interest: The authors declare no conflict of interest.

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