



Classification of Muraenesocidae (Actinopterygii: Anguilliformes) in the East China Sea using Morphological and DNA Barcoding Approaches

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ABSTRACT

The identification of species constitutes the basic step in phylogenetic studies, biodiversity monitoring and conservation. The morphological descriptions and DNA barcoding study about Muraenesocidae in the East China Sea were old, rough and deficient. Morphometric measurements and meristic counts were taken for all collected Muraenesocidae samples in our present study. Teeth characters that are conclusive for the species were consistent with *Muraenesox* and were sufficient for separation from other Muraenesocidae species. The morphological results further suggested that only two Muraenesocidae species belonging to *Muraenesox* inhabit the East China Sea. This study confirms the occurrence of *Muraenesox bagio* (Hamilton, 1822) misreported as *Congresox talabonoides* (Bleeker, 1853) for the first time. *Muraenesox bagio* was diagnosed by: (1) head narrower, interorbital width (IOW) < 10% HL; (2) lateral-line pores before anus (PALL) 33 to 39; (3) dorsal-fin rays before anus (PADR) 49 to 57; (4) vertebrae 131 to 140; (5) red-brown to red color on the pectoral fin; when fresh the body is pale yellow with it being darker above and pale below; the very small black spots are little. In addition, the mitochondrial fragment of 12S rRNA was also sequenced for the purpose of classification. Mean genetic distances within *Muraenesox bagio* (Hamilton, 1822) and *Muraenesox cinereus* (Forsskål, 1775) group were 1.77% and 0.41%, respectively. Group mean distance between *Congresox talabonoides* (Bleeker, 1853) and other species ranged from 11.79% to 51.01%. Species were also confirmed at genetic level. According to present study, *Congresox talabonoides* (Bleeker, 1853) might prefer warm water and live in lower latitude areas. Further sampling is indispensable in order to define their geographic limits.

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Authors' Contribution

ZC conceived and designed experiments. CY, ZC, HW and LY performed all experiments, analyzed the data and wrote the manuscript. ZC and CY arranged financial support.

Key words

Muraenesocidae, The East China Sea, Mis-identification, Marine eels, Actinopterygii.

INTRODUCTION

There are probably 730 fish species in the East China Sea (Yamada *et al.*, 2007; Zhao *et al.*, 2012), constituting about 23% of all marine fish species of China (Chen and Zhang, 2016). They are systematically very diverse, ranging from ancient jawless species (Agnatha) to cartilaginous fishes (Chondrichthyes) and bony fish (Osteichthyes) (Zhu *et al.*, 1963; Zhao *et al.*, 2012). The identification of species constitutes the first basic step for biodiversity monitoring and conservation (Dayrat, 2005). Fish species identification mainly relies on morphometric and meristic characteristics (Strauss and Bond, 1990). However, there are pitfalls in relying primarily on morphology when attempting to identify fishes during

various stages of their development not considered in original treatments or when examining fragmentary, partial or processed remains. Even when intact adult specimens are available, the morphological characteristics used to discern species can be so subtle that identification is difficult even for trained taxonomists (Ward *et al.*, 2009).

It was proposed that the use of DNA barcoding methods can circumvent such a problem since 2003 (Hebert *et al.*, 2003, 2004). The power of DNA barcoding to discriminate closely related species is largely attributable to the abundance of synonymous nucleotide changes (Ward *et al.*, 2005; Ward and Holmes, 2007). Within species, the variation for a targeted DNA barcoding sequence is much lower compared with that between species or cryptic species. As a consequence, species are regularly delineated by a particular sequence or by a tight cluster of very similar sequences (Ward *et al.*, 2009). The reconstruction of phylogenetic relationships based on molecular data in addition to the classical methodologies

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has helped to resolve taxonomic uncertainties for fishes (Hanel and Sturmbauer, 2000; Craig *et al.*, 2001; Herran *et al.*, 2001). In the past two decades, the rise in molecular biological techniques in marine forensic science has violently facilitated the development of accurate taxonomic identification of marine fish species distributed in the East China Sea (Lin *et al.*, 2007; Gao *et al.*, 2011; Chen *et al.*, 2018; Zhang *et al.*, 2019).

Muraenesocidae (pike congers), a widely-distributed family in the coastal waters of China and the Indo-western Pacific, has two recorded species in the East China Sea (Zhu *et al.*, 1963; Zhao *et al.*, 2012, 2016). These two species are *Congresox talabonoides* (Bleeker, 1853) and *Muraenesox cinereus* (Forsskål, 1775). Although many Chinese records about marine fishes have mentioned these two species, Muraenesocidae species taxonomy in the East China Sea is still unclear. To our knowledge, the morphological descriptions about local Muraenesocidae were too old, rough and deficient to identify and distinguish them from each other. For *Muraenesox cinereus* (Forsskål, 1775), the number range of vertebrae, dorsal fin rays before level of anus and lateral line pores before level of anus was too wide, which might include other species (Zhu *et al.*, 1963; Zhao *et al.*, 2012, 2016). The vomerine-teeth difference was the essential character to separate genus *Congresox* and genus *Muraenesox*. However, illustrations of *Congresox talabonoides* (Bleeker, 1853) showed a same tooth pattern with that of *Muraenesox cinereus* (Forsskål, 1775) (Zhu *et al.*, 1963; Zhao *et al.*, 2012, 2016). In some color illustrations, the only difference was that the pectoral-fin color was red brown in *Congresox talabonoides* (Bleeker, 1853) and cinereous in *Muraenesox cinereus* (Forsskål, 1775), respectively (Zhao *et al.*, 2016). The pectoral-fin color was in somewhat conflict with other description of *Congresox talabonoides* (Bleeker, 1853) (Lin *et al.*, 2013). Muraenesocidae species usually migrate annually, occurring off Jeju Island in winter, migrating to the coastal regions of the East China Sea or the Yellow Sea in spring, and returning to the Korea Strait off Japan or Korea again in the fall (NFRDI, 2013; Ji *et al.*, 2015). As a consequence, the species records belonging to three countries should be the same. However, in Japanese systematics, *Muraenesox bagio* (Hamilton, 1822) have been found in abundance, but *Congresox talabonoides* (Bleeker, 1853) has never been recorded (Nakabo, 2002, 2013).

Based on above realities, we hypothesized that there were misidentifications of the greatly-recorded Muraenesocidae in the East China Sea. The first objective of the present study is to supply morphological descriptions and then investigate the record validities of *Muraenesox cinereus* (Forsskål, 1775) and *Congresox talabonoides* (Bleeker, 1853) based on our morphological

evidence. The second objective is to classify all collected Muraenesocidae specimens using DNA barcoding. Our investigation ultimately intended to provide an update on Muraenesocidae composition in the East China Sea.

MATERIALS AND METHODS

Sampling

Samples were collected from Zhoushan, Wenzhou and Fuzhou, from August 2019 to November 2019. All specimens reported here were collected by commercial fishing trawlers and bottom long-line in the East China Sea at depths of 20~100 m. For each station, there are hundreds of specimens suffering a brief on-site examination. Then, the fresh, representative and intact adult specimens covering all recorded types in the past Chinese literatures were collected (Fig. 1).

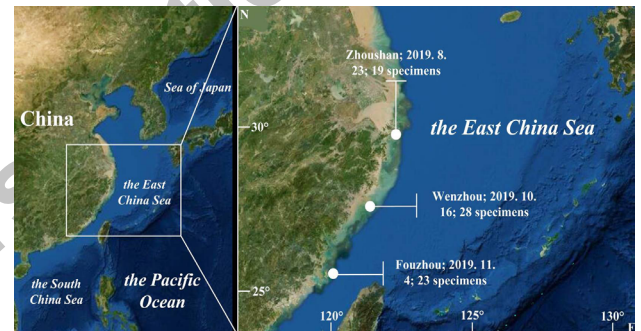


Fig. 1. Sampling location, date, and number of specimens in this study.

All specimens were frozen and immediately transported to laboratory. Identification was carried out based on morphological characteristics (Smith, 1999; Nakabo, 2002; Lin *et al.*, 2013). The brief information of classification was shown in Table I. A piece of muscle tissue was obtained from each specimen and preserved in 95% ethanol. All examined specimens were frozen again and preserved at -20°C .

Morphological study

Measurements were conducted as a straight line (point to point), and dial calipers were used to record to the nearest 0.1 mm. The morphological measurements and meristic counts were utilized as presented by Böhlke (1989) and Lin *et al.* (2013). The following measurement abbreviations are used: TL (total length), PAL (pre-anal length), TR (trunk length), PDL (pre-dorsal length), HL (head length), DGO (body depth at gill opening), GO (gill opening depth), IOW (interorbital width), UJ (length of upper jaw), LJ (length of lower jaw), E (diameter of eye),

Table I.- Information of the Muraenesocidae samples in this study.

Sampling stations	Number of specimens				
	Total number	According to the descriptions in Chinese		According to the descriptions in other languages (English, Japanese, etc.)	
		<i>Muraenesox</i>	<i>Congresox</i>	<i>Muraenesox</i>	<i>Congresox</i>
Zhoushan	19	13	6	19	0
Wenzhou	28	20	8	28	0
Fuzhou	23	19	4	23	0

S (snout length), P (pectoral fin length), VG (ventral width between gill openings), PDL (lateral-line pores before dorsal fin origin), PALL (lateral-line pores before anus), PADR (pre-anal dorsal fin rays), PR (pectoral fin rays), TV (total vertebrae), PAV (pre-anal vertebrae), PDV (pre-dorsal vertebrae), PCV (pre-caudal vertebrae), MVF (mean vertebrae formula). Drawings and photographs were made in this study as well.

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 12S rRNA was amplified using the primers MiFish-U-F: 5'-GTCGGTAAACTCGTGCCAGC-3'; and MiFish-U-R: 5'-CATAGTGGGTATCTAATCCCAGTTTG-3' (Miya *et al.*, 2015). Each polymerase chain reaction (PCR) was performed in a 25 μ L reaction mixture containing 17.5 μ L of ultrapure water, 2.5 μ L of 10 \times PCR buffer, 2 μ L of dNTPs, 1 μ L of each primer (5 μ mol/L), 0.15 μ L of Taq polymerase, and 1 μ L of DNA template. PCR amplification was performed in a Biometra thermal cycler under the following conditions: 5 min of initial denaturation at 95 $^{\circ}$ C; 35 cycles of 25 s at 94 $^{\circ}$ C for denaturation, 25 s at 52 $^{\circ}$ C for annealing, and 25 s at 72 $^{\circ}$ C for extension; and a final extension at 72 $^{\circ}$ C for 5 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 phylogenetic relationships of specimens, 12S rRNA sequences of Muraenesocidae species recorded in China were downloaded from GenBank for comparative analysis (Table II). Sequence of *Anguilla japonica* (Temminck and Schlegel, 1846) was used to root the tree. After aligning by DNASTAR software (Madison, WI, USA), a neighbor-joining (NJ) tree was created. 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.

Table II.- Related 12S rRNA sequences downloaded from NCBI for phylogenetic tree study.

Species	GenBank accession No.
<i>Congresox talabonoides</i> (Bleeker, 1853)	DQ645658
<i>Muraenesox bagio</i> (Hamilton, 1822)	AB049988, LC277765, AP010852
<i>Muraenesox cinereus</i> (Forsskål, 1775)	LC020903, AF417318
<i>Oxyconger leptognathus</i> (Bleeker, 1858)	AF417319, LC327227
<i>Gavialiceps taeniola</i> (Alcock, 1889)	AF417320
<i>Anguilla japonica</i> (Temminck and Schlegel, 1846)	AB038556



Fig. 2. Lateral views of whole body.

RESULTS

Morphological characters

According to their morphological characteristics, only one genus contains 2 species in the East China Sea. The generally morphological features and teeth pattern of them are shown in Figures 2 and 3. Above all, their teeth numbers are abundant, conical-granular and hidden when mouth is closed. Maxillary teeth with several rows extend inward on the eye level. Vomer teeth plate is elongated with three rows; the middle row is enlarged, blade-like, sharp teeth which has a wider base (Figure 3A). Besides, specimens in this study have the following characteristics:

body is moderately elongate, and it is cylindrical anteriorly but becomes compressed posteriorly, with a tapering and flexible tail. Head is acute and elongate. It has an elongate and pointed snout which is slightly narrow and contains a fleshy tip; the upper jaw is curved, and projects beyond the lower jaw with the premaxilla level. The anterior nostril is tubular, laterally and located in the middle between the upper jaw tip and posterior nostril. The posterior nostril is elliptical and located two thirds in front of the eye at the mid-eye level. Rictus extends obviously beyond the posterior margin of the eye. The eye is ellipse and well developed. Gill opening is slit-like, large, located on the lower side, somewhat ventrally, nearly meeting on the end of ventral side, and its height almost reaches half of the body depth. Lateral-line is complete and scale is absent.

The dorsal fin originates slightly before the gill opening, it is well developed and confluent with caudal fin and anal fin; Pectoral fins are well developed. Anus is located before mid-body, about two fifth of the body.

Although all specimens have above similar characters, the two *Muraenesox* species could also be easily separated by the following feature (Figure 3; Table III):

Muraenesox bagio: (1) head narrower, interorbital width (IOW) <10% HL; (2) lateral-line pores before anus (PALL) 33 to 39; (3) dorsal-fin rays before anus (PADR) 49 to 57; (4) vertebrae 131 to 140; (5) red-brown to red color on the pectoral fin (Fig. 3B, the upper one); when fresh the body is pale yellow with it being darker above and pale below (Fig. 2); the very small black spots are little (Fig. 3C).

Table III.- Comparative counts of *Muraenesox bagio* (Hamilton, 1822) and *Muraenesox cinereus* (Forsskål, 1775) from different records.

		Records in this study		Records in references	
		Specimens	Specimens	Lin <i>et al.</i> (2013)	Lin <i>et al.</i> (2013)
		<i>Muraenesox bagio</i> (Hamilton, 1822) (n = 15)	<i>Muraenesox cinereus</i> (Forsskål, 1775) (n = 55)	<i>Muraenesox bagio</i> (Hamilton, 1822) (n = 7)	<i>Muraenesox cinereus</i> (Forsskål, 1775) (n = 76)
Counts	SL (mm)	434-812	380-836	383-855	165-1005
	PDV	7~10 (8.87±0.44)	7~11 (8.15±0.58)	8~9	7~10
	PAV	33~39 (37.32±1.01)	41~48 (45.11±1.49)	37~38	43~48
	TV	131~140 (137.06±2.28)	147~156 (150.94±2.17)	137~138	149~155
	PCV	54~60 (56.87±1.43)	61~67 (64.45±1.61)	56~58	64~66
	MVF	9-37-137	8-45-151	9-38-138	8-45-151
	PDLL	5-8 (6.67±0.94)	4~10 (6.51±1.22)	6~7	4~8
	PALL	33~39 (36.16±1.34)	41~47 (43.34±1.24)	35~37	40~46
	PADR	49~57 (52.82±2.11)	66~78 (72.51±2.28)	50~54	66~80
	PR	16~17 (16.94±0.17)	16~18 (16.96±0.41)	17	15~18
As % TL	PAL	36.25~41.71 (38.56±2.28)	36.16~49.40 (40.29±2.75)	36.55~42.78	36.21~46.92
	Tail	53.66~62.58 (56.83±2.97)	54.12~63.02 (55.45±2.38)	57.22~63.45	55.68~63.92
	TR	23.34~25.81 (24.20±1.09)	17.66~27.25 (23.48±2.14)	22.99~23.94	19.22~29.27
	PDL	13.20~14.75 (14.03±0.65)	12.20~14.34 (13.69±0.50)	14.06~14.36	12.01~15.28
	HL	13.94~16.36 (14.91±1.10)	12.52~16.93 (14.32±1.46)	14.06~14.62	12.84~16.53
DGO	4.49~6.45 (6.05±0.94)	4.26~6.74 (6.06±0.81)	4.62~5.06	3.96~7.28	
As % DOG	GO	34.38~55.88 (44.74±8.76)	21.21~44.44 (33.58±5.11)	-	-
As % HL	IOW	8.63~9.74 (8.97±0.39)	11.75~17.39 (13.26±1.18)	9.23~10	11.39~20.37
	UJ	46.90~51.00 (48.94±1.81)	45.25~52.33 (48.51±1.99)	48.87~49.99	46.04~55.41
	LJ	39.02~45.33 (41.43±2.91)	38.02~54.00 (42.96±3.81)	44~45.94	39.73~52.43
	E	9.68~13.00 (11.28±1.17)	9.46~15.43 (11.82±1.48)	11.33~13.21	9.99~15.54
	S	25.81~32.33 (21.18±1.91)	24.68~33.99 (28.21±2.10)	26.07~29.23	26.96~32.83
	P	30.44~43.55 (38.11±2.84)	31.00~43.66 (40.01±3.02)	29.82~35.73	30.13~44.03
	VG	6.67~9.68 (8.33±1.24)	5.26~9.33 (6.87±1.13)	-	-

Table IV.- Genetic distances within (on the diagonal, bold font) and between (below the diagonal, normal font) species.

	<i>Muraenesox bagio</i>	<i>Muraenesox cinereus</i>	<i>Congresox talabonoides</i>	<i>Oxyconger leptognathus</i>	<i>Anguilla japonica</i>	<i>Gavialiceps taeniola</i>
<i>Muraenesox bagio</i>	0.0177					
<i>Muraenesox cinereus</i>	0.0426	0.0041				
<i>Congresox talabonoides</i>	0.1339	0.1179	-			
<i>Oxyconger leptognathus</i>	0.1589	0.1894	0.2725	0.0000		
<i>Anguilla japonica</i>	0.1950	0.2000	0.2420	0.2365	-	
<i>Gavialiceps taeniola</i>	0.3504	0.3539	0.5101	0.3272	0.2259	-

Muraenesox cinereus: (1) head broader, interorbital width (IOW) >10% HL; (2) lateral-line pores before anus (PALL) 41 to 47; (3) dorsal-fin rays before anus (PADR) 66 to 78; (4) vertebrae 147 to 156; (5) cinereous to black color on the pectoral fins (Fig. 3B, the lower one); when fresh it is light gray to gray, with a deep color above (Fig. 2); the very small black spots are numerous (Fig. 3D).

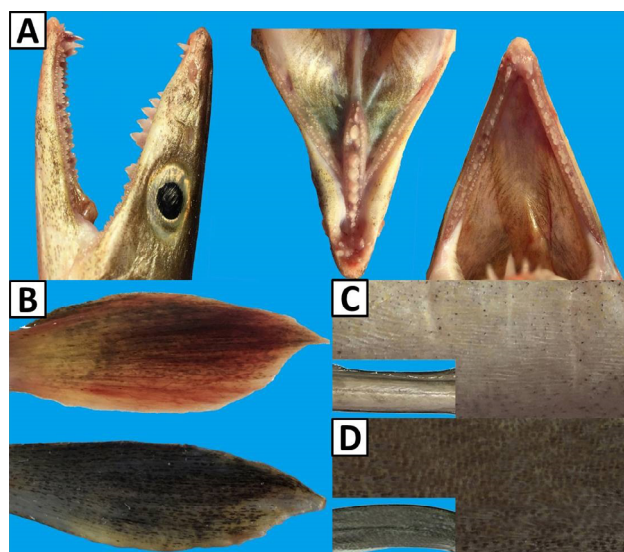


Fig. 3. Some characteristic difference between *Muraenesox bagio* and *Muraenesox cinereus* in the East China Sea.

Molecular analyses

For each species, two specimens of each population randomly chosen were sequenced. Twelve 172-bp-long sequences of 12S rRNA gene fragments were obtained. After combined the downloaded sequences of Muraenesocidae, a total of 22 sequences were used for analysis. A neighbor-joining phylogenetic tree was constructed using MEGA 5.0 (Fig. 4). The 12S rRNA sequences of specimens from the East China Sea clustered in three groups, and 3 haplotypes were defined. All haplotype sequences were

submitted to GenBank with the following accession numbers: MN811203-MN811205. The haplotype 1 (Hap-4) was shared by 6 specimens of *Muraenesox cinereus* (Forsskål, 1775). The haplotype 3 (Hap-3) were shared by 1 specimens of *Muraenesox bagio* (Hamilton, 1822) from Zhoushan. The remaining specimens of *Muraenesox bagio* (Hamilton, 1822) shared the haplotype 2 (Hap-2). Table IV shows the genetic distances among all species. The mean distance among all species was 14.98%. Within mean group distance of *Muraenesox bagio* (Hamilton, 1822) and *Muraenesox cinereus* (Forsskål, 1775) were 1.77% and 0.41%, respectively. Genetic distance between our 12 *Muraenesox* specimens and *Congresox talabonoides* (Bleeker, 1853) downloaded from NCBI was >11.79%, which indicated that our specimen couldn't be *Congresox talabonoides* at all.

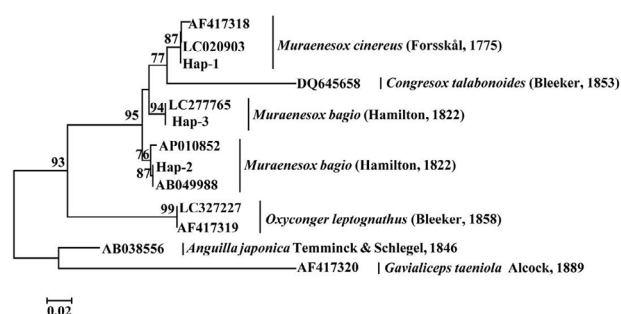


Fig. 4. Phylogenetic tree based on neighbor-joining analysis of 12S rRNA sequence. Numbers above branches indicate neighbor-joining bootstrap percentages. Only bootstrap values of >60% are shown in the above NJ tree.

DISCUSSION

The morphological characters of specimens used in this study were photographed, counted and compared with previously representative records on Figures 2, 3 and Table III. The specimens in the East China Sea were characterized by: blade-like rather than needle-like teeth

on the vomer. This decisive phenotypic trait was consistent with the typical *Muraenesox* specimens, and ruled out the possibility of genus *Congresox* these samples belonging to Smith (1999) and Nakabo (2013). According to our present study, it was concluded that previous records of *Congresox talabonoides* (Bleeker, 1853) were the mis-identification of *Muraenesox bagio* (Hamilton, 1822) in reality, and the latter was a new record in the East China Sea (Zhu *et al.*, 1963; Zhao *et al.*, 2012, 2016).

Congresox talabonoides (Bleeker, 1853) was initially recorded as *Muraenesox talabonoides* (Bleeker, 1853) (Smith, 1999; Lin *et al.*, 2013; Zhao *et al.*, 2016), both genus being characterized by: body long, compressed along tail; snout very pointed, mouth terminal, large, extending well beyond eye; teeth always large, prominent, especially in front sharp, multi-serial on jaws and typically in 3 rows on vomer; gill opening a vertical or oblique slit in front of pectoral fin; no spines in fins; dorsal fin begins before gill opening; pectoral fins always present; no pelvic fins; no scales. By reviewing all morphological characteristics including our present results, it could be obviously found that *Muraenesox* and *Congresox* were very similar. Almost all morphological characteristics were much overlapped (Smith, 1999; Lin *et al.*, 2013; Nakabo, 2013). These might be the reason why *Muraenesox bagio* (Hamilton, 1822) from the East China Sea was misidentified as *Congresox talabonoides* (Bleeker, 1853) by native ichthyologists.

DNA barcoding was recognized as an effective and reliable method for species identification (Hebert *et al.*, 2003; Domingues *et al.*, 2013; Chen and Gao, 2017). The impossibility that specimens belonged to *Congresox talabonoides* (Bleeker, 1853) was also demonstrated from genetically phylogenetic relationship. We identified that the sequence of *Congresox talabonoides* (Bleeker, 1853) from NCBI (DQ645658) was distinguished from other species by distance = 0.1179~0.5101. Within mean group distance of *Muraenesox bagio* (Hamilton, 1822) and *Muraenesox cinereus* (Forsskål, 1775) were 1.77% and 0.41%, respectively. The interspecific distance was respectively about 7.56 and 28.76 times larger than intraspecific distance. Together, both the morphological and genetic analysis strongly supported the validity of *Muraenesox bagio* (Hamilton, 1822) and nonexistence of *Congresox talabonoides* (Bleeker, 1853) in the East China Sea.

It was reported that *Congresox talabonoides* (Bleeker, 1853) was widely distributed in the coastal waters of China (Zhu *et al.*, 1963; Jin, 2006; Tang, 2006; Zhao *et al.*, 2012, 2016). We have also tried our best to collect more specimens from China seas. But it seemed that fishermen could only see the occurrence of *Congresox talabonoides* (Bleeker, 1853) in the East China Sea. This species

might prefer warm water and live in lower latitude areas. Further domestic and overseas specimen collection is also indispensable in order to define its clearly geographic limits.

Accurate identification of fish is essential and would assist in managing fisheries for long-term sustainability, and improve ecosystem research and conservation. This nature will require careful morphological analysis from expert taxonomists before any final recommendations can be made (Ward *et al.*, 2005; Hajibabaei *et al.*, 2007; Gross, 2012). Mitochondrial sequence divergences are strongly linked to the process of speciation, and DNA barcoding and morphological analysis should go hand-in-hand. We hope this study will not only promote the sustainable exploitation, biodiversity conservation and fisheries management of *Muraenesox* distributed in the East China Sea but also contribute to species identification within this family.

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Statement of conflict of interest

There are no conflicts of interest and authors are solely responsible for the contents and writing of the paper.

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