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Redescription and DNA Barcoding of *Sillago indica* (Perciformes: Sillaginidae) from the Coast of Pakistan

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ABSTRACT

This study deals with the redescription of *Sillago indica* based on seventy-two specimens measuring (139.2-192.4 mm SL), obtained from the fish market in Weifang City, China, in March 2014, these fishes were originally captured in the coastal waters of Pakistan. The meristic counts as: dorsal fin rays XI, I, 20~22; anal fin rays II, 21~23; pectoral fin rays 16~17; pelvic fin rays I, 5; caudal fin rays 16~17; gill rakers first arch 3~4+7~8=10~12; vertebrae 33~35 (mostly 34). The swim bladder with two anterior extensions extending forward to basioccipital on both sides above auditory capsule; two posterior extensions extending into haemal funnel beyond posterior end of body cavity (the roots of two posterior extensions are non-adjacent and two posterior extensions are not well-knitted); a single duct-like process originating from ventral surface of swim bladder (between the roots of two posterior extensions) and reaching urogenital opening. Due to the similarities and complexities of morphological characters used in traditional taxonomy, taxonomic confusion has arisen in *Sillago* concerning the nomenclature. Therefore, the fragment of cytochrome oxidase subunit I (COI) gene of mitochondrial DNA was sequenced for the classification of *Sillago* species in future. The mean genetic distance within the species *S. indica* was 0.2%, net genetic distances between *S. indica* and other six species of the genus *Sillago* ranged from 17.1% to 22.2%. Comments are made on some of the characters to more fully characterize the species and for phylogenetic studies.

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Authors' Contributions
TG designed and conducted the study. JX and NS performed the experiments and analyzed data. RJM M helped in manuscript preparation. JX finalized the article.

Key words
***Sillago indica*; morphological characters; swim bladder; DNA barcoding.**

INTRODUCTION

Sillago indica McKay, Dutt and Sujatha, 1985, is commercially important species and widely distributed in the east and west coasts of India and generally confused with other *Sillago* species in commercial catches (McKay, 1985). Due to the similarities and complexities of morphological characters, elaborated shape of the swim bladders is essential to identify the species (McKay, 1992). *S. indica* is described as belonging to the subgenus *Sillago* with two posterior extensions (Kaga and Ho, 2010), not one as shown in the original illustration (McKay, 1985, who placed this species in his subgenus *Parasillago*).

The mitochondrial cytochrome oxidase I gene (COI) varies noticeably between species and very little between the individuals of a given species (Gross, 2012). Therefore, a fragment of COI gene, as DNA barcoding (Hebert *et al*., 2003), has proven to be extremely effective at discriminating species (Domingues *et al*., 2013; Puckridge *et al*., 2013; Ming *et al*., 2015), discovering new-recorded and new species (Gao *et al*., 2011; Qin *et al*., 2013), uncovering cryptic species (Hajibabaei *et al*., 2007; Zemlak *et al*., 2009), identification of ichthyoplankton (Bian *et al*., 2008; He *et al*., 2011). In the present study, seventy-two specimens of *S. indica* were collected from the market of Weifang, China, which were caught in the coast of Pakistan. The aim of this study was to redescribe *S. indica* based on correct morphological characters and DNA barcoding. The results will highlight the need for caveats when identifying *Sillago* species and be helpful to fishery management, biodiversity conservation, and sustainable exploitation of this species.

MATERIALS AND METHODS

Sample collection

In this study, 72 individuals were examined, obtained from fish market in Weifang, China, in March 2014. The specimens were originally captured from the coastal waters of Pakistan. All individuals were identified based on morphological characteristics (McKay, 1985, 1992). The body color and pigmentation were pictured in fresh fish and all measurements were made on preserved specimens. The shapes of swim bladders were pictured and freehand sketched. For the genetic study, a piece of muscle tissue was obtained from each individual and preserved in 95% ethanol or directly extracted from frozen samples. All specimens examined were frozen and preserved at the Fishery Ecology & Marine Biodiversity Laboratory, Fisheries College, Zhejiang Ocean University (Zhoushan).

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Table I.- GenBank accession numbers of related COI sequences and information of *Sillago* samples in this study.

Species	Sampling sites	Sample size	Accession no.
S. japonica[a]	Jimo (Shandong Province, China)	5	KM350221 KM350222 KM350223 KM350224 KM350225
S. aeolus[a]	Wenzhou (Zhejiang Province, China)	4	KM350212 KM350213 KM350214 KM350215
S. indica[a]	Pakistan	10	KM350229 KM350230 KM350231 KM350232 KM350233 KM350234 KM350235 KM350236 KM350237 KM350238
S. sihama[a]	Dongshan (Fujian Province, China)	5	KM350216 KM350217 KM350218 KM350219 KM350220
S. ingenuua[a]	Dongshan (Fujian Province, China)	3	KM350226 KM350227 KM350228
S. sinica[b,c]	Korea, China	4	KC708229, HQ389256, HQ389254, HQ389242
S. bassensis[b]	Australia	4	HM131481, HM131482, HM131483, HM131484

Data sources: a, this study; b, Gao *et al*., 2011; Bae *et al.*, 2013

Morphological analysis

Methods for taking measurements and counts followed Kaga *et al*. (2010). Institutional abbreviations followed Fricke and Eschmeyer (2011). Counts included: dorsal fin spines and rays, anal fin spines and rays, pectoral fin rays, pelvic fin spines and rays, caudal fin rays, gill rakers on the first gill arch, vertebrae, scales in lateral line, scales above lateral line, and scales below lateral line. Measurements included: standard length, head length, postorbital length, snout length, eye diameter, interorbital width, caudal peduncle depth, caudle peduncle length, first dorsal fin base, second dorsal fin base, anal fin base, pectoral fin length, pelvic fin length, body width. All measurements were made with calipers to the nearest 0.1 mm. Comparative data were taken from Kaga *et al*. (2010).

After measurements were taken, both sagittas were removed from each specimen and placed in 1.5 ml plastic tubes containing distilled water. After ultrasonic cleaning, the otoliths were baked in an oven for 24 hour at 50°C until a constant mass and weight to the nearest 0.01 mg were achieved. Lateral-view digital images of otoliths were taken with a Nikon SMZ800 microscope equipped with a Nikon digital sight DS-Fi1 (Tokyo, Japan).

Phylogenetic analysis

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method. Mitochondrial (mt) DNA cytochrome oxidase subunit I (COI) was amplified using primers
L5956-COI: 5'-CACAAAGACATTGGCACCCT-3';
H6558-COI: 5'-CCTCCTGCAGGGTCAAAGAA-3'
(Gao *et al*., 2011). PCR was carried out in 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 µM), 0.15 µL of *Taq* polymerase, and 1µL of DNA template. The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: 5 min initial denaturation at 95°C, and 35 cycles of 45 s at 94°C for denaturation, 45 s at 50°C for annealing, and 45 s at 72°C for extention, and a final extention at 72°C for 10 min. PCR product was purified with a Gel Extraction Mini Kit. The purified product was used as the template DNA for cycle sequencing reactions performed using BigDye Terminator Cycle Sequencing Kit, and bi-direction sequencing was conducted on an ABI Prism 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers used for sequencing as those for PCR amplification.

Lasergene software (Lasergene, Madison, WI, USA) was used for the sequence comparison. MEGA 5.0 (Tamura *et al*., 2011) was used to analyze the sequences and reckon the net genetic distances under the Kimura 2-parameter (K2P) model. Ten COI haplotypes of *S. indica* with other relevant sequences obtained in the present study were submitted to GenBank (Table I).

RESULTS

Morphological characters

The measurements and meristic counts of 72 *S. indica* individuals were examined, total length from 160.6~222.6 mm. General body features are shown in Figure 1, morphometric data are given in Table II.

Body elongate, with a long conical snout, slightly compressed, head tapering with terminal mouth. Mouth small, with band of small villiform teeth, opening slightly oblique. Upper jaw with small canines forming tooth band, becoming narrower posteriorly. Lower jaw with small canines, forming tooth band anteriorly, width same as upper jaw tooth band, tooth band gradually becoming narrower posteriorly, terminating in one row. Palatine and tongue toothless. Vomer with two to three rows of canine teeth. Eye moderate, its margin covered with adipose eyelid. Interorbital region flat. Nostrils situated anterior to upper margin of eye; anterior nostril tubular with flap

anteriorly, posterior nostril lacking flap. Two large slit-like sensory pores open onto tip of snout, just above upper lip. Posterior margin of preopercle slightly serrated. Opercle with single weak spine posterodorsally. Body covered with small or moderate sized ctenoid scales, those of cheeks and opercles in 2-3 rows all cycloid except for occasional ctenoid scale posteriorly; lateral line scales simple. Gill rakers on first arch pointed, with small irregular spines; two tooth plates on hypobranchial portion of arch.

Table II.- Meristic counts and proportional measurements of *Sillago indica.*

	Paratypes[a,b] n=3	Specimens[c] n=72
Standard length (mm, SL)	126.5-159.4	139.2-192.4
Counts		
Dorsal fin rays	XI,I,21-22	X-XI,I,20-22
Anal fin rays	II,22-23	II,21-23
Pectoral fin rays	16-17	16-17
Pelvic fin rays	I,5	I,5
Caudal fin rays	—	16-17
Scales in lateral line	68-70 (70-71)	68-71
Scales above lat. line	6	5-6
Scales below lat. line	11-12	10-12
Gill rakers first arch	3+8=11	3-4+7-8=10-12
Vertebrae	34	33-35 (mostly 34)
Measurements (%, SL)		
Head length	29.4-30.1	27.5–32.4
Postorbital length	—	10.0-13.1
Snout length	12.0-13.2	11.1-14.2
Eye diameter	5.2-6.2	4.8-6.9
Interorbital width	5.4-6.3	4.8-7.7
Caudal peduncle depth	7.1-7.7	6.6-8.9
Caudle peduncle length	—	8.9-11.7
First dorsal fin base	17.9-20.0	19.3-23.7
Second dorsal fin base	34.2-36.4	33.9-38.0
Anal fin base	35.1-37.1	31.8-37.1
Pectoral fin length	16.0-17.0	14.4-19.1
Pelvic fin length	15.6-16.0	14.1-17.9
Body width	13.7-15.8	13.2-18.8

Data sources: a, McKay, 1985, 1992; b, Kaga and Ho, 2012; c, present study

Color when fresh, upper surface of trunk yellowish-brown, grading to silver on abdomen. A faint mid-lateral stripe usually present. Cheek and opercle with fine black dots, belly and lower sides may be densely dotted, almost blackish. Dorsal fin membranes dusted with black dots. Pectoral fin yellowish, no blotches on base. Anal fin yellowish, interradial membranes dusted with black dots. Caudal dusted with black, posterior margin blackish and lower lobe densely black.

Over time in preservation specimens may variable in coloration, especially of fins, were included: body and head yellowish brown overall, melanin increase, scales with fine black dots, dorsal and abdominal portions separated by a mid-lateral stripe. The fins turn yellow, dorsal and anal fin membranes dusted with black dots; posterior margin of caudal fin blackish and lower lobe densely blackish, pigment zone of caudal fin showed an inverted "M" shaped. In paratype, body and head dark brown overall, no obvious dark black band on midline of body. Abdomen with fine black dots. Snout dark gray, lower half of opercle blackish, and cheek with fine black dots. Dorsal and anal fin membranes dusted with black dots. Lower lobe of caudal fin is dark gray.

Dorsal fins X-XI, I, 20~22; anal fin II, 21~23; pectoral fin 16~17; pelvic fin I, 5; caudal fin 16~17. lateral line scales 68~71, 5~6 above, 10~12 below; gill rakers first arch 3-4+7-8; vertebrae 34. As percentages of standard length (%, Table II): head length 27.5~32.4; snout length 11.1~14.2; eye diameter 4.8~6.9; interorbital width 4.8~7.7; caudle peduncle length 8.9~11.7; first dorsal fin base length 19.3~23.7; second dorsal fin base length 33.9~38.0; anal fin base length 31.8~37.1; pectoral fin length 14.4~19.1; pelvic fin length 14.1~17.9; body width 13.2~18.8. As percentages of head length (%): snouts length 40.1~46.9, eye diameter 16.6~22.9, interorbital width 16.7~23.7, postorbital length 34.5~42.3. As percentages of caudle peduncle length (%): caudal peduncle depth 63.0~79.6.

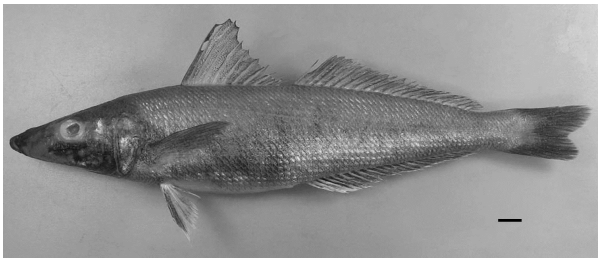

Fig. 1. *Sillago indica*, 191.4 mm SL, from Pakistan. Bar 10 mm.

The characteristic of swim bladder conforms to the description by Kaga and Ho (2012), with a slightly modified (Fig. 2). Swim bladder large, with two anterior extensions extending forward to basioccipital on both sides above auditory capsule; two posterior extensions extending into haemal funnel beyond posterior end of body cavity, the roots of two posterior extensions are non-adjacent and two posterior extensions are not well-knit. A single duct-like process originating from ventral surface of swim bladder and reaching urogenital opening, the origin of the duct-like process is at the terminal of swim bladder and between the roots of two posterior extensions; a subextension of duct-like process is present, small but complex, connect with a sanguineous vesicle

close to vertebra, with unknown function. An anterolateral extension present on each side of swim bladder, branching into anterior and posterior subextensions: anterior one comprising a short, simple blind tubule; posterior one kinky, long and thin, situated along abdominal wall and reaching to the roots of two posterior extensions, relatively. Eight or nine lateral processes extending from entire lateral surface of main body of swim bladder into musculature, anterior three stout and horn-like, posterior five or six rather small and triangular in shape. The posterior subextensions of the swim bladder are ventrally adherent to the lateral processes but do not communicate with them.

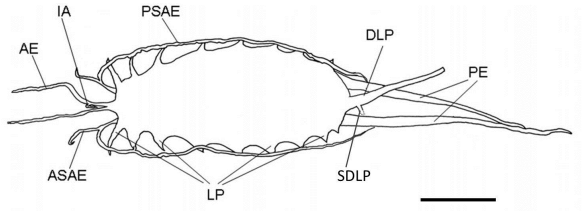

Fig. 2. Swim bladder of *Sillago indica*, 191.4 mm SL. ASAE, anterior subextension of anterolateral extension; AE, anterior extension; LP, lateral process; DLP, duct-like process; IA, irregular appendage; PSAE, posterior subextension of anterolateral extension; PE, posterior extension; SDLP, subextension of duct-like process.

The morphological description of otoliths of *S. indica* (Fig. 3B) is as follows: Roughly triangular, the edge of dorsal is smooth, with one slight gap at the posterior end; the ventral edge is irregularity with two distinct protuberances on the ventral edge, the anterior of otolith incline to the ventral slightly. Other features are similar to *S. sihama* (Fig. 3A): There is a main groove throughout the otolith (sulcus), with an opening at both ends. The surface of the non-groove side is bright, with radial stripes and tumor-like rimes in the middle.

Sequence analysis

Five *Sillago* species in the present study combined some *S. sinica* and *S. bassensis* sequences downloaded from GenBank, totally thirty-five sequences were used in the analysis. All haplotype sequences were submitted to GenBank with the accession numbers in Table I. After sequence alignment, a 601 bp fragment of the COI gene was obtained. There were no indels and insertions, and 204 variable sites were observed in the 35 specimens of 7 *Sillago* species. Net genetic distances (K2P) within and between species are shown in Table III. Net distances between pairs of the 7 *Sillago* species ranged 0.171-0.222, and values of differentiation between *S. indica* and *S. ingenuua*, *S. aeolus*, *S. sihama*, *S. japonica*, *S. sinica* and *S. bassensis* were 0.215, 0.208, 0.171, 0.219, 0.215 and 0.211, respectively. The NJ tree was constructed based on the K2P model. Bootstrap values were tested by 1000 replicates. *Sillaginodes punctatus* was initially chosen as the outgroup to root the tree (Fig. 4).

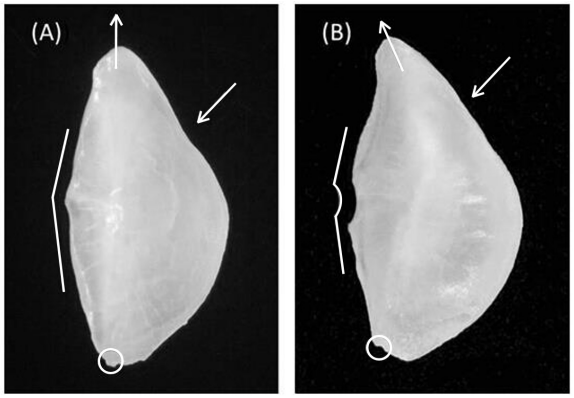

Fig. 3. Otoliths of *Sillago sihama* (A) and *Sillago indica* (B), the differences were marked in white.

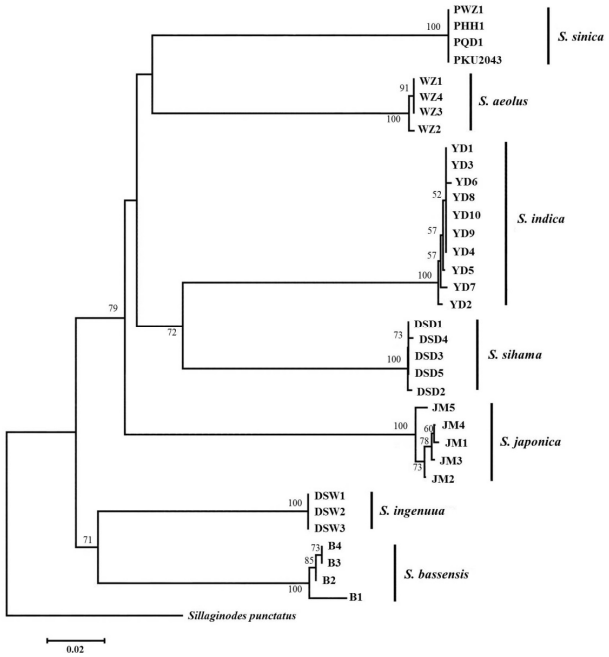

Fig. 4. Neighbor-joining (NJ) tree for cytochrome oxidase subunit I (COI) gene sequences of 8 Sillaginidae species. The NJ tree was constructed under the K2P model using *Sillaginodes punctatus* as the outgroup. Bootstrap support values of > 50% from 1000 replicates are shown.

Table III.- Net genetic distances (K2P) within (on the diagonal) and between (below the diagonal) the 7 *Sillago* species.

	S. japonica **n=5**	***S. sinica*** **n=4**	***S. aeolus*** **n=4**	***S. indica*** **n=10**	***S. bassensis*** **n=4**	***S. sihama*** **n=5**	***S. ingenuua*** **n=3**
S. japonica	**0.006**						
S. sinica	0.210	**0.000**					
S. aeolus	0.219	0.194	**0.002**				
S. indica	0.219	0.215	0.208	**0.002**			
S. bassensis	0.212	0.222	0.195	0.211	**0.009**		
S. sihama	0.204	0.210	0.180	0.171	0.215	**0.001**	
S. ingenuua	0.202	0.203	0.198	0.215	0.153	0.196	**0.000**

Table IV.- Comparisons of meristic characters of seven *Sillago* species.

	Dorsal fin	**Anal fin**	**L.I**	**L.tra**	**Gill rakers**	**Vertebrae**
S. japonica [a,b]	XI,I,21~23	II,22~24	70~73	3~4/8~10	4~5/9~10	34~35
S. sinica [b,c]	X-XI,I,20~22	II,21~23	75~79	7~8/10~12	2~4/6~8	37~39
S. aeolus [a,b]	XI,I,18~20	II,17~19	67~72	7~8/9~10	3/7~8	33~34
S. indica [c]	X-XI,I,20~22	II,21~23	68~71	5~6/10~12	3~4/7~8	33~35
S. bassensis [a,b]	X-XII,I,18~19	II,18~20	66~73	5~6/10~14	6~7/13	33~35
S. sihama [a,b]	XI,I,20~23	II,21~23	66~72	5~6/10~12	3/8~9	34
S. ingenuua [a,c]	XI,I,17	II,17	66~70	3~4/8~9	3/9~10	33

Data sources: a, McKay, 1985, 1992; b, Xue, 2010; c, present study

DISCUSSION

In this study, seventy-two specimens based on morphological characters and DNA barcoding were examined. The morphological characters were counted and measured, then compared with the holotype, which is deposited in the Zoological Survey of India, Calcutta (ZSI; collected by K. Sujatha, June 8, 1979). Based on our observations, all characters are in agreement with prior records of this species (Table II). Although some do not exactly fall into the range recorded by McKay (1985) and Kaga and Ho (2012), which is reasonable given the differences in growth rate and sample size. All countable characters conforms the original description by McKay (1985), however, there were two exceptional specimens in vertebrae: one is 33 and the other 35, not 34 as original description. This is likely because the number of holotype is weak, the original range of vertebrae is not broad to cover all *S. indica*. In this study, based on our counts, the range for vertebrae should be 33-35. McKay studied 6 specimens from Weifang markets imported from Pakistan and found that the abdominal vertebrae number 12 to 13 (13-4-17 (5) and 12-4-18 (1)), not 14 as in *S. sihama* from Pakistan (14-4-16 (6), 14-5-15(4), 14-3-17(1)); the first haemal arch is very thin and easily broken in *S. indica*. McKay (1985) counted 238 *S. sihama* with 14 abdominal vertebrae and none with 12 or 13. The ranges for measurable characters in this study generally were expansive, deviations may made by experimenters and the same reasons affect the countable characters. Comparisons of meristic characters of 7 *Sillago* species in different records was shown below (Table IV). The darker coloration of *S. indica* is a fairly reliable distinguishing character that makes recognition in mixed catches easy, but identification should be based on the vertebrae, swim bladder and otolith where possible. In the present study, the melanin on the trunk of frozen samples increases, the fins turn yellow, lower lobe of caudal fin densely blackish and pigment zone of caudal fin showed an inverted "M" shape. The mid-lateral dark stripe on *S. indica* can be very dark to black or diffuse and interrupted as 7 to 9 elongate blackish spots.

The swim bladders of tested paratypes are nearly identical to that reported description of *S. indica* by Kaga and Ho (2012). It was confirmed that the swim bladder of *S. indica* was very similar to that of *S. sihama*, which reflected the close genetic relationship between this two congeneric species. Similarly, on the anterior extension, and on the posterior subextension of the anterolateral extension of the swim bladder, were completely matched. In comparison of the swim bladder of *S. sihama*, anterolateral extension from the swim bladder of *S. indica* may be thinner, with simpler structure; the roots of two posterior extensions are non-adjacent and two

posterior extensions are not well-knit, in its natural state, there is a lacuna between the two posterior extensions; the origin of the duct-like process is at the terminal of swim bladder and between the roots of two posterior extensions. However the swim bladder of *S. sihama*, the roots of two posterior extensions are adjacent and two posterior extensions are in close; the origin of the duct-like process is in front of the terminal of swim bladder and the joint of roots of two posterior extensions. Moreover, a subextension of duct-like process (SDLP) was present in *S. indica* and *S. sihama*, small but complex, connect with a sanguineous vesicle close to vertebra, but the function still needs further research. The swim bladder could not be observed completely because the internal organs were not removed from the abdominal cavity.

Otoliths are calcareous structures that act as statoliths in the internal ear of fish (Su, 2005) and are well known for their time-recording structure that is widely used in studies to assess age (Lowerre-Barbieri *et al*., 1994; Álvarez *et al*., 2007). In addition, otolith microscopic examination and measurements are often used to differentiate fish stocks (Tuset *et al*., 2006). Differences among otoliths in different species reflect varying deposition rates which are mainly affected by metabolic rates and other genetic factors. Therefore, otoliths can be used as aids to identify species (Qin *et al*., 2013). However, the otoliths of *S. indica* were resembled with *S. sihama* with exception of the number of the distinct protuberances on the ventral edge and the gap or protuberance at the posterior end.

On examination of the genetic distance data for these 35 specimens (Table III), *S. indica* and *S. sihama* show the least divergence, separating from each other at an average of 17.1%. *S. indica* and *S. sihama* may be a pair of sister species based on our comparison between these two in morphology, swim bladder, otoliths and COI sequence. As an indicator of speciation, Hebert *et al*. (2004) proposed the '10×rule', whereby barcoded individuals are flagged as possible another species if they diverge by 10 times or more the average intraspecific variability of the group. A different approach was taken by Ward *et al*. (2009), who analysed barcode data from about 1000 fish species and showed that at a level of 2% distance or greater, individuals were much more likely to be congeneric than conspecific. We identified that *S. indica* and other *Sillago* species are distinguished by d = 0.171~0.219. All other pairwise divergence among species exceeded d = 0.150, and the highest values were observed between *S. bassensis* and *S. sinica* (d = 0.222). The mean evolutionary distance within the species *S. indica* was 0.2%, use of either the 10× or 2% rule suggests that the genetic distance between groups was significantly higher than the average genetic distance within the group, which indicated that the COI gene used as a barcode of *S. indica* was effective at identifying *Sillago* species.

Accurate identification of fish is essential and would enable us in retail substitutions of species to be detected, assist in managing fisheries for long-term sustainability, and improve ecosystem research and conservation. Resolution of cases of this nature will require careful morphological analysis from expert taxonomists before any final recommendations can be made (Ward *et al*., 2005). Mitochondrial sequence divergences are strongly linked to the process of speciation, DNA barcoding and morphological analysis should go hand-in-hand. The data presented here would aid more information and explicit species taxonomy and avoid numerous misidentification and erroneous distributional records within *Sillago* genus.

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