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DNA BARCODING APPLICATION OF MITOCHONDRIAL COI GENE TO IDENTIFY SOME FISH SPECIES OF FAMILY GOBIIDAE IN VIETNAM

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Abstract. The family Gobiidae is a relatively high abundance family of coastal fish with about 2000 species in 210 genera described. In Vietnam, the study on Gobiidae is very complex due to the large number of species, small size and high morphological variation which makes difficulties in identification and classification. In this study, the DNA barcoding technique has been used through mitochondrial cytochrome oxidase I (COI) gene to classify 30 specimens of Gobiidae from three coastal areas (North, Central and South) in Vietnam to evaluate the effectiveness compared to the morphological classification method. Results showed that 26 species belonging to 21 genera were determined by the COI barcode while 19 species (17 genera) were determined when using morphological method. *Mahidolia mystacina* was new recorded in Vietnam. The DNA barcodes of COI gene developed in this study could be useful for estimating phylogenetic diversity as well as other studies of gobiids in terms of conservation, management and utilization of fisheries resources in Vietnam. In addition, the results showed the high potentiality in using COI barcode to identify marine fish.

Keywords: DNA barcoding, COI, Gobiidae, Vietnam.

INTRODUCTION

Family Gobiidae consists of more than 2,000 species and occupies over 200 genera [1, 2]. In Vietnam, the Gobiidae has five subfamilies: Amblyopinus, Gobiinae, Gobionellinae, Oxudercinae and Tridentigerinae [3]. In particular, most studies on Gobiidae in Vietnam have been conducted in small geographic areas, for example: 9 species were recorded in Vinh Long province [4]; 11 species in Tra Vinh province [5]; 59 species in the Mekong Delta [6]; 59 species in Soc Trang [7];... However, these data were mainly based on morphological characteristics, while the Gobiidae is generally characterized by small size, a high capacity to adapt to environmental challenges and diversification of habitats. They show remarkable morphological and ecological variability in different habitats, therefore identification of Gobiidae based on morphometrics experiences many difficulties.

Presently, DNA barcoding is considered a useful tool in the classification of organisms with high accuracy [8]. Molecular biology has contributed to addressing taxon identification and phylogenetic relationship questions, mitochondrial cytochrome oxidase subunit I (COI) (mtDNA) has been widely recognized and used in the identification and classification of new species of animals [9], and COI has

been used to successfully classify a series of taxa [10–12], to identify Gobiidae with 114 species in South Korea [13], 11 species in India [14], and 7 species in Vietnam.

Therefore, research to applicate new techniques in organism classification is very important. In this study, the 30 samples of Gobiidae which were collected from three coastal areas (North, Central and South) of Vietnam have been classified by two methods: Comparative morphology and DNA barcoding technique (mitochondrial gene of cytochrome oxidase I - COI) to evaluate effectiveness of the DNA barcode technique in the fish identification, and contribute to improving the efficiency and quality of researches on organism classification in Vietnam.

MATERIALS AND METHODS

Sampling and morphological identification. A total of 30 specimens of Gobiidae were collected based on random sampling at the fish markets in Quang Ninh - Hai Phong (North), Ninh Thuan (Central) and Ca Mau - Kien Giang (South) in the 2017 and 2018. Then, the fish sample was coded, photographed and fin tissue was cut. The fin tissue samples were preserved in eppendorf tubes with alcohol 96% and stored at -20^{o}C until analysis.

All fish specimens were identified to species based on morphological characteristics according to the taxonomic system of Rainboth (1996) [15], Nakabo et al., (2002) [16].

DNA barcoding identification

DNA extraction, PCR amplification and sequencing. Total DNA was extracted from the tissue of each individual fish using "G-spinTM Total DNA Extraction Kit (iNtRON)" following the manufacturer's instructions. The 650 bp mitochondrial COI fragment was amplified with the primers Fish F (5'- TCA ACC AACC AC AAA GAC AT TGG C AC-3') and Fish R (5' -TAGAC T TC TGG GTGG CC AA AGAATC A-3') [17, 18]. The PCR reaction was performed with total volume of 25 µl including: 10 ng DNA template, 2.5 µl Buffer (1X), 5 µl DNA sample, 1 µl per primer (10 µM), 0.5 µl dNTP (10 µM), 0.125 µl Dream Taq Polymerase (5 U/µl) and distilled water to the final volume. Biorad thermocyclers (Icycler) were used under the following temperature program: Initial denaturation 94^{o}C for 5 min, followed by 35 cycles of 95^{o}C for 45 seconds, 50^{o}C for 45 seconds, 72^{o}C for 1 minute; and final extension at 72^{o}C for 7 minutes. PCR products were electrophoresed on 1.5% agarose gel stained with 2 µl SYBR® Gold Nucleic Acid Gel Stain, and DNA bands were visualized under a UV transilluminator. The results are recorded using the GelDoc image analysis system. One to two µl of PCR products was purified using a PCR clean up system kit "MEGAquick- spinTMPlus Total Fragment DNA Purification Kit (iNtRON)", and then nucleotide sequencing followed the principle Dye-labelled dideoxy terminator (Big Dye Terminator v.3.1, Applied Biosystems) with each of the same primers used in PCR reactions at the following programs: 96^{o}C for 30 s, 50^{o}C for 30 s and 60^{o}C for 4 min. Products were analyzed using an ABI Prism 3.700 DNA Analyzer (Applied Biosystems).

DNA barcoding identification and phylogenetic analysis. Sequences were initially aligned using the sequence editor BioEdit 7.2.6.1 [19], clustered in Clustalw X software [20]. The resulting sequences were referred to databases by the Basic Logical Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/) program on GenBank and BOLD System to identify species. Genetic distance was built using the Test Neighbor-joining algorithm with a bootstrap value of 1000 times for the sample (high reliability: > 85%, average reliability: 65–85%, low reliability: < 65%). The Bayesian Information Criterion (BIC) -based model was selected to build interrelated relational trees and be corrected using MEGA 7 software. Several sequences on GenBank (table 1) have been used to compare fish species in this study.

RESULTS AND DISCUSSION

Species identification based on morphological method. The results of identification of 30 fish specimens according to the morphological method were shown in table 1 and table 2, in which 16 specimens were classified to species including 14 species belonging to 4 subfamilies, 5 specimens were

identified to genera (DOS 05842, DOS 05843, DOS 05834, DOS 05006, DOS 06009 belong to *Oligolepis*, *Stenogobius*, *Apocryptodon*, *Yongeichthys* and *Acentrogobius*, respectively), and 9 specimens were undetermined (DOS03694, DOS04864, DOS04865, DOS03691, DOS04808, DOS05837, DOS05840, DOS05841, DOS06013).

Table 1. Results of classification of 30 goby specimens by comparative morphology and DNA barcoding technique

Specimens	Subfamilies	Morphology method	DNA barcoding method		
		Species	Species	Similarity	GenBank number
DOS05839	Gobiinae	*Acentrogobius chlorostigmatoides*	*Acentrogobius chlorostigmatoides* (Bleeker, 1849)	99%	JX193727.1
DOS06009		*Acentrogobius* sp. *	*Acentrogobius chlorostigmatoides* (Bleeker, 1849)*	99%	JX193727.1
DOS03689		*Amblygobius phalaena*	*Amblygobius phalaena* (Valenciennes, 1837)	100%	KP194353.1
DOS05446		*Bathygobius fuscus*	*Bathygobius fuscus* (Rüppell, 1830)	99%	KF265065.1
DOS04111		*Cryptocentrus leptocephalus*	*Cryptocentrus leptocephalus* (Bleeker, 1876)	99%	FJ583293.1
DOS04817		*Favonigobius reichei*	*Favonigobius reichei* (Bleeker, 1854)	99%	KY371540.1
DOS04901		*Glossogobius aureus*	*Glossogobius aureus* (Akihito & Meguro, 1975)	99%	KC789535.1
DOS05835		*Glossogobius giuris*	*Glossogobius giuris* (Hamilton, 1822)	99%	MG680939.1
DOS03690		*Psammogobius biocellatus*	*Psammogobius biocellatus* (Valenciennes, 1837)	99%	KU944841.1
DOS04902		*Psammogobius biocellatus*	*Psammogobius biocellatus* (Valenciennes, 1837)	99%	KU944841.1
DOS03820		*Parachaeturichthys polynema*	*Parachaeturichthys polynema* (Bleeker, 1853)	100%	KY315375.1
DOS05685		*Valenciennea puellaris*	*Valenciennea puellaris* (Tomiyama, 1956)	99%	HQ536635.1
DOS05447		*Pseudogobius javanicus*	*Pseudogobius javanicus* (Bleeker, 1856)	99%	KU692802.1
DOS05006		*Yongeichthys* sp.*	*Yongeichthys criniger* (Valenciennes, 1837)*	99%	KT894736.1
DOS06013		sp. 09*	***Mahidolia mystacina* (Valenciennes, 1837)**	99%	HQ536694.1
DOS04289		*Oligolepis acutipennis*	*Oligolepis acutipennis* (Valenciennes, 1837)	99%	HQ654727.1
DOS05842		*Oligolepis* sp.*	*Oligolepis acutipennis* (Valenciennes, 1837)*	99%	HQ654727.1
DOS05843		*Stenogobius* sp.*	*Stenogobius gymnopomus* (Bleeker, 1853)*	96%	KU692904.1
DOS03694	Gobionellinae	sp. 01*	*Oxyurichthys* sp. *01**	96%	
DOS04864		sp. 02*	*Oxyurichthys* sp. *02**		
DOS04865		sp. 03*	*Oxyurichthys* sp. *03**	95%	KY176548.1
DOS03691		sp. 04*	*Oxyurichthys* sp. *04**	92%	
DOS04808		sp. 05*	*Oxyurichthys* sp. *05**	90%	
DOS05840		sp. 07*	*Stigmatogobius pleurostigma* (Bleeker, 1849)*	99%	KU692906.1
DOS04537	Amblyopinae	*Amblyotrypauchen arctocephalus*	*Amblyotrypauchen arctocephalus* (Alcock, 1890)	100%	KY371128.1
DOS05833		*Trypauchen vagina*	*Trypauchen vagina* (Bloch & Schneider, 1801)	99%	KY315379.1
DOS03693		*Amblyotrypauchen arctocephalus**	*Trypauchen vagina* (Bloch & Schneider, 1801)*	99%	KY315379.1
DOS05834	Oxudercinae	*Apocryptodon* sp. *	*Pseudapocryptes elongatus* (Cuvier, 1816)*	100%	10475.1
DOS05837		sp. 06*	*Boleophthalmus boddarti* (Pallas, 1770)*	99%	KY754676.1
DOS05841		sp. 08*	*Parapocryptes serperaster* (Richardson, 1846)*	99%	KT965855.1

Note: * newly recorded species or species were renamed by the DNA barcoding technique. Species name in bold word was new record for the goby in Vietnam and its GenBank number was MK119259.1.

Table 2. Number of species and genera between sampling regions and subfamilies

Subfamilies / Regions	Gobidae		Gobionellinae		Amblyopinae		Oxudernae		Total	
	Species	Genera	Species	Genera	Species	Genera	Species	Genera	Species	Genera
North	3	3	0	0	1	1	0	0	4	4
Central	8	8	1	1	1	1	0	0	10	10
South	3	3	2	2	1	1	1	1	7	7
Total *	13	12	3	2	2	2	1	1	19	17

Note: *: Total number of species or genera identified.

The species composition in this study consisted of four subfamilies: Gobiinae, Gobionellinae, Amblyopinae and Oxudercinae (tables 1, 2). In which, Gobiinae had the highest number of specimens and number of species with 13 species belonging to 12 genera that were determined from 15 specimens, and 2 specimens (DOS06009, DOS05006) were identified to *Acentrogoobius* and *Yongeichthys*, respectively; Oxudercinae had the lowest species composition with 1 species; Gobionellinae consisted of 3 species belonging to 2 genera that were determined from three specimens; Amblyopinae had 2 species of 2 genera that were determined from three specimens.

According to the sampling area, 10 species belonging to 10 genera in 3 subfamilies and 3 undetermined specimens were recorded in the Central area. 7 species belonging to 7 species in 4 subfamilies and 4 undetermined specimens were recorded in the South area. 4 species belonging to 4 genera in two subfamilies and 2 underestimated specimens were recorded in the North area.

Species identification based on DNA barcoding of COI gene. A total of 30 COI sequences were generated from 30 gobiid fish specimens collected from three coastal areas of Vietnam. Sequence length ranged from 502 bp to 700 bp (mean 660 bp). The average base composition was 23.8% adenine (A), 28.4% cytosine (C), 17.6% guanine (G) and 30.2% thymine (T). A total of 207 polymorphic sites accounted for 41.2%. Nucleotide diversity index of Vietnamese Gobiidae species is 0.22%. The length of 502 bp of all 30 sequences was used for the analysis and construction of the phylogenetic tree (fig. 1).

The results of comparing the nucleotide sequences of 30 fish samples with GenBank data through BLAST and BOLD systems were shown in tables 2, 3. In total, 26 species belonging to 21 genera in 4 subfamilies (Gobiinae, Gobionellinae, Amblyopinae and Oxudercinae) were recorded. In which, 25 specimens were successfully identified to species level with a high similarity (99–100%), and 5 specimens were identified to genera level because of no data available on GenBank. This provided the potential for cryptic species and new sequences for GenBank.

Between the 4 subfamilies, Amblyopinae had the lowest number of species with 2 species belonging to 2 genera; Oxudercinae had three species belonging to three genera; Gobionellinae consisted of 5 specimens that were identified to genera and 4 specimens that were identified to 3 species; Gobiinae had the maximum number of species with 13 species belonging to 12 genera from 15 specimens.

Based on sampling regions, 4 subfamilies were obtained in South region, 3 subfamilies were obtained in the North and Central regions, except the Oxudercinae subfamily. In which, 13 species of 12 genera were obtained in the Central region; 5 species of 4 genera were found in the North region and 11 species of 11 genera were collected in the South region.

Table 3. Number of species and genera between regions and subfamily

Subfamilies / Regions	Gobidae		Gobionellinae		Amblyopinae		Oxudernae		Total	
	Species	Genera	Species	Genera	Species	Genera	Species	Genera	Species	Genera
North	2	2	2	1	1	1	0	0	5	4
Central	8	8	4	3	1	1	0	0	13	12
South	4	4	3	3	1	1	3	3	11	11
Total*	13	12	8	4	2	2	3	3	**26**	**21**

Note: *: Total number of species or genera was identified.

Discussion

Characteristics of fish species composition. Tables 2, 3 show that *Oxyurichthys* had the highest number of species with 5 species, accounting for 19.2%, followed by *Glossogobius* with 2 species (7.7%), the remaining genera had only one species (3.8%). Specially, *Mahidolia mystacina* was a new record for the Goby fish in Vietnam based on Fishbase (accessed on 24/9/2018), Animals of Vietnam in Volume 2 [21] and List of Marine Fish in Vietnam [22]. Although the number of fish samples in this study was not large (30 samples), but the number of obtained species was relatively diverse compared to other regions in Vietnam as well as in some studied regions in the world (table 4). 26 species were obtained in this study as many as the number of species recorded in the Anambas and Natuta archipelagos, but it was less than that in the Gulf of Thailand (28 species) and Weh Indonesia (53 species).

Table 4. Number of species, genera of Gobiidae in some studied regions

Location	Genera	Number of species	References
Southern Vietnam (1992)	16	21	Mai Dinh Yen (1992) [3]
Mekong delta (1993)	10	11	Truong Thu Khoa et al., (1993) [23]
Mekong delta (2013)	32	58	Tran Dac Dinh et al., (2013) [6]
Soc Trang coast (2014)	17	22	Diep Anh Tuan (2014) [7]
Nha Trang bay		34	Do Thi Cat Tuong (2015) [24]
Truong Sa islands		13	Nguyen Nhat Thi et al., (2004) [25]
Ha Long bay		9	Nguyen Van Quan (2004) [26]
Phu Yen		3	Nguyen Van Long (2013) [27]
The Gulf of Thailand		28	Satapoomin (2000) [28]
Weh islands		53	Allen and Werner (2002) [29]
Anambas and Natuta islands		26	Adrim et al., (2004) [30]
Some coastal areas of Vietnam (2018)	**17**	**19**	This study - *morphological identification*
		26	This study - *DNA Barcoding*

Phylogenetic relationships based on mitochondrial COI gene. The results in fig. 1 show that species and genera had a very distinct division with high bootstrap indexes (> 85%), which also reflected the effectiveness and high accuracy of the identification by the COI (table 1, fig. 1).

The phylogenetic tree of the studied fish in fig. 1 was divided into 3 main groups. Therein, **Group 1** included 10 species belonging to genera *Acentrogobius, Amblygobius, Bathygobius, Cryptocentrus, Glossogobius, Psammogobius, Parachaeturichthys, Pseudogobius* and *Yongeichthys* in Gobiinae (subfamily) with typical morphological features such as short snout, developed ventral fins; most of them had 2 rows of first dorsal fin rays and second rays of soft fin, many color dot on the body. Two species (*G. aureus and G. giuris*) in the same genus (*Glossogobius*) in this study (fig. 1) had also been confirmed in previous studies [31]. Lucke et al., (2014) also pointed out that the two genera (*Bathygobius* and *Glossogobius)* had a long genetic distance, although they belonged to the same subfamily, which was also presented in the results of this

study (fig. 1). **Group 2** was divided into two subgroups: ***Subgroup 2.1*** included 3 species belonging to *Amblyotrypauchen* and *Trypauchen* in the subfamily Amblyopinae, and ***subgroup 2.2*** included 3 species belonging to *Pseudapocryptes, Boleophthalmus, Parapocryptes* in the subfamily Oxudercinae. **Group 3** was divided into 3 subgroups: ***Subgroup 3.1*** included 5 specimens of genus *Oxyurichthys*, ***subgroup 3.2*** included 3 species of genera *Favonigobius, Valenciennea, Mahidolia* in Gobiinae subfamily, **subgroup 3.3** included 4 species of 3 genera *Oligolepis, Stigmatogobius , Stenogobius* in the subfamily Gobionellinae.

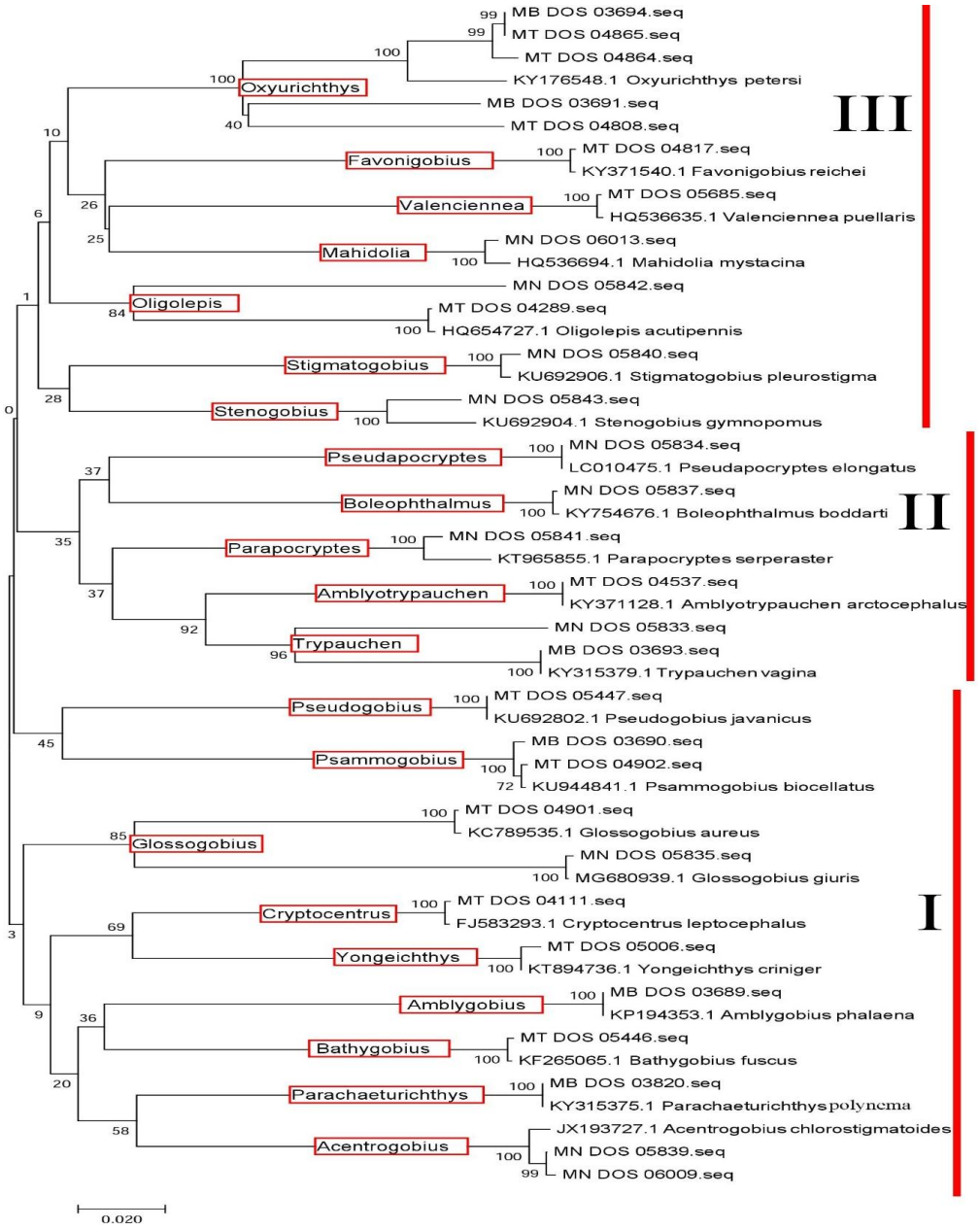

Fig. 1. The phylogenetic tree was based on the COI gene with the GTR + G + I model following the Neighbor Joining method, with a bootstrap value of 1000 times

Efficiency between two classification methods. A total of 26 species, 21 genera, 4 subfamilies (Gobiinae, Gobionellinae, Amblyopinae and Oxudercinae) belonging to the Gobiidae family were obtained from 30 goby fish specimens through two classification methods. In which, the DNA barcoding method (COI gene) identified 26 species (21 genera) while the morphology method only identified 19 species of 17 genera. In particular, some specimens which could not be identified or identified incorrectly according to the morphological method had been identified to species and revised by DNA barcoding method. Therein, eight fish specimens (DOS06009, DOS05006, DOS06013, DOS05842, DOS05843, DOS05840, DOS05833, DOS05834) were identified to species or their names were revised (table 1). Therein, DOS05833 specimen was re-identified as *Trypauchen vagina* (100% similarity) instead of *Amblyotrypauchen arctocephalus,* the DOS05834 specimen was identified as *Pseudapocryptes elongatus* (with 100% similarity) in place of the unidentified species (*Apocryptodon* sp.*)* The specimens (DOS05840, DOS05837, DOS05841, DOS06013) were identified as species *Stigmatogobius pleurostigma, Boleophthalmus boddarti, Parapocryptes serperaster* and *Mahidolia mystacina* (with 99% similarity), respectively. 5 specimens (DOS 03694, DOS 04864, DOS 04865, DOS 03691, DOS 04808) were identified to *Oxyurichthys* genera with similarity from 90% to 96%.

The morphological classification has the advantages as fast and economic method, many references, no requirement on modern equipments and expensive chemicals but it requires much experience from experts. Specially, the traditional morphological method for species identification are constrained by phenotypic plasticity, life stage specific identification cues, small size, often cryptic ecologies and the occurrence of new species. Meanwhile, DNA barcoding method overcomes the limitations of morphological method, showing the effectiveness, high accuracy and resolving taxonomic ambiguities in many cases. For example, square-head climbing perch and wild strain had difference in head shape and variety of morphological parameters but they had the same COI gene sequence [32, 33]. Two goby species of *B. butis* and *B. humeralis* were the same species [34]. Or salmon *Oncorhynchus mykiss* had two types with two different names (rainbow trout and steel head) with difference of morphological and life cycle characteristics but they were the same species [35, 36]. The sequences of 16S, Cyt b and D-loop genes showed that the two salmon strains differed only by one or two nucleotide positions. In contrast, some species had the same morphology but they were different species based on the COI gene, for example: In Africa, there were seven different species from the fish which was known as "Acará" through sequences of COI and 12S rRNA genes [37]. Thus, the above examples and the present results reconfirmed that the DNA barcoding method of the mitochondrial COI gene is highly effective in identifying, classifying and assessing the emergence of species, including the Gobiidae. In addition, species identification by morphological method should be combined with the DNA barcoding method in some cases, may even require the use of two or more different genes in difficult cases as five samples remaining at the genera level.

CONCLUSION

30 specimens of Gobiidae collected from three coastal areas (North, Central and South) in Vietnam were identified, 26 species belonging to 21 genera by the COI barcoding method while 19 species (17 genera) were identified by morphological method. *Mahidolia mystacina* was newly recorded in Vietnam. The DNA barcodes of COI gen developed for 26 species in this study could be useful for estimating phylogenetic diversity as well as other studies of gobiids in conservation, management and utilization of fisheries resources in Vietnam. Once again, the results showed the high potentiality in using COI barcode to identify marine fish in Vietnam.

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