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DNA barcoding for identification of some fish species (Carangidae) in Vietnam coastal area

Pham The Thu[1,*], Nguyen Manh Linh[1], Nguyen Van Quan[1], Pham Van Chien[1], Dao Huong Ly[1], Le Ba Hoang Hiep[2]

[1]*Institute of Marine Environment and Resources, VAST, Vietnam*
[2]*VNU University of Sciences, Vietnam National University, Hanoi, Vietnam*
[*]E-mail: thupt@imer.vast.vn

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Abstract
Carangidae family has got about 148 species belonging to 32 genera. In Vietnam, Carangidae is of high commercial value and playing an important role in the ecosystem. In the context Vietnam has received yellow card for seafood since Nov. 2017 by the EU, in which one of the main reasons was related to the restriction of traceability. In this study, DNA barcoding technique of mitochondrial cytochrome oxidase I (COI) gene was used to classify 56 specimens of Carangidae from three coastal areas (Northern, Central and Southern) in Vietnam to evaluate the effectiveness compared to the morphological classification method. Results showed that 21 species belonging to 16 genera were determined by the COI barcode while 18 species (16 genera) were determined when using morphological method. *Seriola quinqueradiata* and *Trachinotus anak* were newly recorded in Vietnam. From 56 sequences with 660 bp of mtDNA (COI), total 27 haplotypes were detected; haplotype diversity (h) and nucleotide diversity (π) were 0.903 ± 0.00060 and 0.14%, respectively. The DNA barcodes of COI gene of 21 species in Carangidae which were developed in this study could be used as a basis for comparison and traceability of their products. In addition, the results showed the high potentiality in using COI barcode to identify Carangidae fish in Vietnam.

Keywords: DNA barcoding, COI, Carangidae, Vietnam.

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INTRODUCTION

The European Union (EU) has penalized a yellow card for Vietnam's seafood from 10/2017 to date, partly due to the traceability [1]. Therefore, the research and establishment of a database to serve the traceability of Vietnamese seafood origin are very important. On the other hand, the fish species identification has been mainly based on the comparative morphological method. However, it is not always possible to identify a specimen based on its phenotypic characteristics because some species show highly morphological variations according to their habitat, which may not be available (eg specimens were processed), immature and ungrown (eg fish larvae or juvenile). Moreover, morphological method requires a lot of experience, expertise in morphology [2–5].

Recently, DNA barcoding of the mitochondrial cytochrome oxidase I (COI) gene (mtDNA) has been widely used in animal identification studies and is being considered as a global standard for species identification with several advantages like small amount of biological samples needed, applicability for all life stages, fast analysis, high accuracy and differentiation among phenotypically alike species [6–11].

Carangidae is among the most economically important coastal pelagic fishes of the world and this family is one of the bonefish families with 148 species belonging to 32 genera [12], in 2010 they were exploited with a production reaching 1,556,578 tons [13]. In Vietnam, the Carangidae family is a main part of the commercial fishing industry [14, 15]. Despite the high economic value and important ecological role, the research on Carangidae in Vietnam is still limited and difficult in the identification [16–18].

Therefore, in this study, 56 fish samples of the Carangidae family collected from 3 coastal areas (Northern, Central, and Southern) in Vietnam were classified by two methods: Morphological form DNA barcoding and comparison of mitochondrial COI genes, to evaluate the effectiveness of genetic barcode techniques and to build DNA barcode data for Vietnamese Carangidae fish family, contributing to improving efficiency and quality of biological classification studies and service of traceability of commercial fishery products of Vietnam.

MATERIALS AND METHODS

Sample collection

A total of 56 samples of Carangidae were collected randomly at three localities along coast of Vietnam: Quang Ninh - Hai Phong provinces (Northern locations - N) with 44 samples, Ninh Thuan province (Central location - C) with 9 samples, Ca Mau - Kien Giang provinces (Southern locations - S) with 3 samples in 2017 and 2018. Each specimen was immediately photographed and the tissue was sampled. Tissue samples of approximately 1 to 3 g of fin clips were cut, stored in 95% ethanol and frozen at −20^{o}C before the extraction of DNA.

Morphological identification

All fish specimens were identified to species based on morphological characteristics according to the taxonomic system of Rainboth (1996) [19], Nakabo et al., (2002) [20].

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 AGA ATC A-3") [21, 22]. The PCR the reaction was performed with a 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 a 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 30s, 50^{o}C for 30s and 60^{o}C for 4 min. Products were analyzed using an ABI Prism 3.700 DNA Analyzer (Applied Biosystems, IGR).

Data processing and building phylogenetic relationships

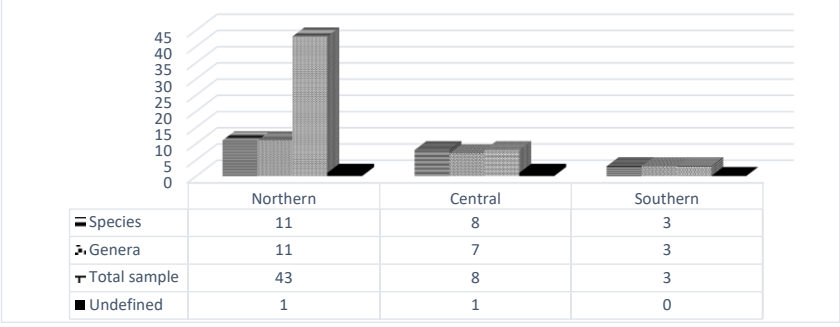
The gene sequences were analyzed by BioEdit software 7.2.6.1 [23], clustered in Clustalw X software [24], then the nucleotide sequence was included in the BLAST program (Basic Local Alignment Search Tool) on GenBank and BOLD System to compare and identify species. Characteristics of DNA genetic barcodes of samples were determined by the number of haplotype (k), the number of polymorphic sites (s), haplotype diversity (d) and nucleotide diversity (π), mutant number (n) through software DNAsp v4.0 [25]. Genetic distance was built by algorithm Test Neighbor-joining with bootstrap (BT) value (high confidence level: > 85%; average confidence level: 65–85%; low confidence level: < 65%) repeating 1,000 times the test sample. The Bes (Bayesian Information Criterion) BIC model was selected to build the interrelated relation tree and calibrated with MEGA X software. Some sequences on GenBank (table 1) were used to compare with the sequences of fish in this study.

RESULTS AND DISCUSSION

Species identification based on the morphological method

The classification results of 56 fish samples according to morphological methods are shown in fi. 1 and table 1. In which, 54 samples were identified as 18 species of the Carangidae family and 2 samples were identified as *Alepes, Gnathanodon*.

According to the sampling area, 11, 8 and 3 species belonging to 11, 7 and 3 genera were recorded in the Northern, Central and Southern locations, respectively. One specimen was undetermined to the species in the Northern and Central locations (fig. 1).



	Northern	Central	Southern
Species	11	8	3
Genera	11	7	3
Total sample	43	8	3
Undefined	1	1	0

Fig. 1. The number of species and genera of the Carangidae family among three sampling locations was determined by morphological method

Species identification based on DNA barcoding of COI gene

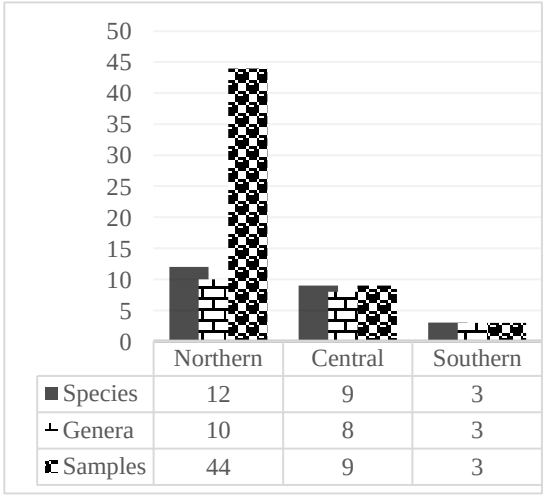
A total of 56 COI sequences were generated from 56 Carangidae fish specimens collected from three coastal areas of Vietnam. The sequence length ranged from 522 bp to 700 bp (mean 660 bp) and there was not any codon, insert or delete in the sequences. The average base pair composition was 23.8% adenine (A), 29.8% cytosine (C), 16.6% guanine (G) and 29.8% thymine (T). Genetic characteristics of 56 sequences were identified: A total of 158 polymorphic sites accounted for 41.2%; number haplotype (n), haplotype diversity (h),

nucleotide diversity (π), mutation number (η) and polymorphic site (s) index were 27, 0.903 ± 0.0006, 0.14%, 347 and 282, respectively. In which, 18, 9 and 3 haplotypes were recorded from 44, 9 and 3 samples of the Carangidae populations at Northern, Central and Southern locations, respectively.

The length of 522 bp of all 56 sequences was used to analyze and construct phylogenetic trees (fig. 2). The results of comparing the nucleotide sequences of studied fish samples with GenBank data through BLAST and BOLD Systems were shown in table 1.

In total, 21 species belonging to 16 genera in the Carangidae family were recorded with high similarity (99–100%). In which, the species variation among genera was 1 to 2 (fig. 2), the highest number of species was 3 species (accounting for 13.7%) in Scomberoides genus, 2 species (accounting for 9.1%) was in Alepes, Carangoides, Trachinotus genera each and only 1 species (accounting for 7.7%) was in the rest genera each (Atute, Decapterus, Megalaspis, Parastromateus, Selar, Selaroides, Seriolina, Seriola, Trachurus, Uraspis, Alectis).

According to the sampling area, 12; 9 and 3 species belonging to 10; 8 and 3 genera were recorded in the Northern, Central and Southern locations, respectively.



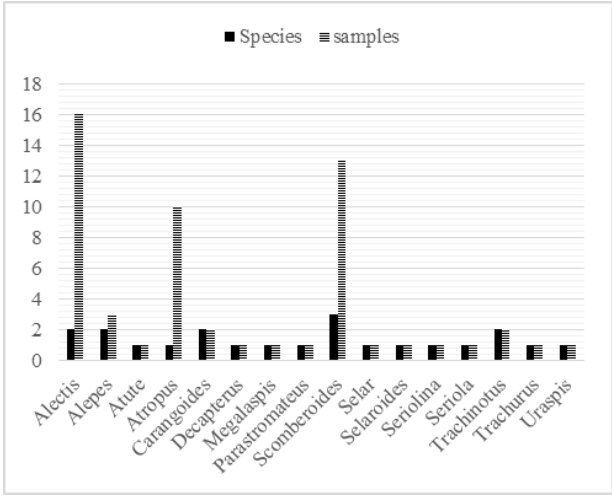


Fig. 2. The number of species and genera of the Carangidae family among three sampling locations was determined by barcoding method

Total 21 species belonging to 16 genera, 4 subfamilies (Caranginae, Naucratiniae, Scomberoidiniae, Trachinotiniae) of the Carangidae family were determined from 56 fish samples by both research methods (table 1). In which, the barcoding method (COI gene) recorded 21 species (16 genera) while only 18 species (16 genera) were recorded by the morphological method. In particular, some specimens which could not be identified or was identified incorrectly according to the morphological method has been identified or revised to species by DNA barcoding method. Therein, nine fish specimens (DOS03650, DOS04767, DOS05768, DOS04342, DOS05257, DOS04738, DOS03661, DOS03655, DOS04639) were identified to species by morphological methods but their names were revised accurately with a high similarity rate (table 1): *Alepes kleinii* (99%), *Atule mate* (100%), *Atropus atropos* (99%), *Decapterus maruadsi* (99%), *Scomberoides tol* (99%), *Selaroides leptolepis* (99%), *Seriolina nigrofasciata* (99%), *Trachurus japonicus* (100%), *Uraspis uraspis* (99%), respectively. Two samples (DOS04241, DOS03656) were only identified to genus level (*Alepes* sp., *Gnathanodon* sp.) by morphological method while they were identified to species (*Alepes kleinii*_99%, *Alepes vari*_99%), respectively.

Table 1. The classification result of 56 Carangidae specimens by comparative morphological method and DNA barcoding method

Specimens	Morphological method	DNA barcoding method			
	Species	Genera	Species	Similarity (%)	GenBank number
DOS03649 HL11.(1–4) HL12.(1–2) TY16.(1–9)	*Alectis indicus*	Alectis	*Alectis indicus* (Rüppell, 1830)	99	NC037050.1
DOS03650	*Alepes djedaba*	Alepes	*Alepes kleinii* (Bloch, 1793)	99	KT326328.1
DOS04241	*Alepes sp.*				
DOS03656	*Gnathanodon* sp.		*Alepes vari* (Cuvier, 1833)	99	KF714896.1
DOS04767	*Caranx tille*	Atute	*Atule mate* (Cuvier, 1833)	100	KU317883.1
TY10.(1–9)	*Atropus atropos*	Atropus	*Atropus atropos* (Bloch & Schneider, 1801)	99	KY371167.1
DOS05768	*Carangoides hedlandensis*		*Atropus atropos* (Bloch & Schneider, 1801)	99	KY371167.1
DOS03652	*Carangoides malabaricus*	Carangoides	*Carangoides malabaricus* (Bloch & Schneider, 1801)*	100	KJ174514.1
DOS03653	*Carangoides oblongus*		*Carangoides ferdau* (Forsskål, 1775)*	99	KF714902.1
DOS04342	*Trachurus japonicus*	Decapterus	*Decapterus maruadsi* (Temminck & Schlegel, 1843)	100	KY570761.1
DOS03658	*Megalaspis corddyla*	Megalaspis	*Megalaspis cordyla* (Linnaeus, 1758)*	99	KM522836.1
DOS03659	*Paratromateus niger*	Parastromateus	*Parastromateus niger* (Bloch, 1795)	100	MF737197.1
DOS04666 HL9.(1–10)	*Scomberoides commersonnianus*	Scomberoides	*Scomberoides commersonnianus (*Lacepède, 1801)*	100	KU499732.1
DOS05521	*Scomberoides lysan*		*Scomberoides lysan* (Forsskål, 1775)	99	DQ885125.1
DOS05773			*Scomberoides tol* (Cuvier, 1832)	99	KU535574.1
DOS05774	*Selar crumenophthalmus*	Selar	*Selar crumenophthalmus* (Bloch, 1793)	99	KF009661.1
DOS05257	*Alepes djedaba*	Selaroides	*Selaroides leptolepis* (Cuvier, 1833)	99	KM522839.1
DOS04738	*Naucrates ductor*	Seriolina	*Seriolina nigrofasciata* (Rüppell, 1829)*	99	KU535575.1
DOS03660	*Seriola quinqueradiata*	Seriola	*Seriola quinqueradiata* (Temminck & Schlegel, 1845)*	99	KU168712.1
DOS03661	*Trachinotus blochii*	Trachinotus	*Trachinotus anak* (Ogilby, 1909)	99	KP641582.1
DOS04005	*Trachinotus blochii*		*Trachinotus baillonii* (Lacepède, 1801)*	100	KU535576.1
DOS03655	*Decapterus maruadsi*	Trachurus	*Trachurus japonicus* (Temminck & Schlegel, 1844)	100	HM180926.1
DOS04639	*Uraspis helvola*	Uraspis	*Uraspis uraspis* (Günther, 1860)*	99	KU578093.1

Note: The bold names were newly recorded species or were renamed by the DNA barcoding method. "*" were species belonging to LC list of IUCN Red Book accessed on November 22, 2018. HL11.(1÷4) included four specimens (HL11.1; HL11.2; HL11.3; HL11.4).

Discussions

Characteristics of fish species composition

Based on small number of specimens (56), total 21 species belonging to 16 genera which were recorded was diverse compared to some coastal areas in the western Pacific (table 2). Specially, the number of genera was similar to that in some near-coastal areas such as the northern Gulf of Thailand (16 genera) and in the Indo-Malay (16 genera) and Taiwan (22 genera) (table 2), a total of 50 species in Vietnam [26] and 148 species in the world have been recorded. On the other hand, from the results of COI gene analysis, two species of *Seriola quinqueradiata* (Temminck & Schlegel, 1845) * and *Trachinotus anak* (Ogilby, 1909) were newly records for the Carangidae fauna in Vietnam based on FishBase (accessed on 23/10/2018), Animals of Vietnam in Volume 19 [27] and Atlas of common coral reef fishes in Vietnam [28], hence, the total number of species of the Carangidae family in the Vietnam was increased to 52. Eight of 21 fish species in present study were on the LC list of IUCN Red Book. The result in present study could be considered a good data for the management of fisheries resources and biodiversity conservation.

Table 2. Number of species, genera of Carangidae in some studied areas

Location	Genera	Species	References
Mai Giang estuary	3	3	Hoang Ngoc Thao et al., (2015) [29]
Can Gio mangroves	8	9	Tong Xuan Tam et al., (2014) [30]
Van Phong Bay (Khanh Hoa)	13	19	Tran Thi Hong Hoa et al., (2014) [14]
Marine fish in Vietnam	21	46	Nguyen Huu Phung et al., (1995) [31]
Marine coral reef fish in Vietnam	16	26	Nguyen Nhat Thi et al., (2005) [32]
Tam Giang - Cau Hai lagoon	9	12	Nguyen Van Hoan et al., (2012) [33]
Taiwan	22	54	Lin et al., (1999) [34]
Indo-Malay sea	18	36	Jaafa et al., (2012) [35]
Fishes of northern gulf of Thailand	16	24	Tomohiro et al., (2013) [36]
Some species of Carangidae in Vietnam	16	21	This study

Phylogenetic relationships based on mitochondrial COI gene

The results in fig. 3a, 3b show that the 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. 3a, 3b).

The phylogenetic tree of the studied fish in fig. 3 was divided into 4 main groups corresponding to the indicator colour lines for 4 subfamilies in the Carangidae. Therein, **Group 1** included 3 species belonging to subfamily Scomberoidae indicated in blue with the highest bootstrap value (99%), including *Scomberoides commersonnianus, Scomberoides lysan, Scomberoides tol*; **Group 2** included two species of *Trachinotus baillonii* and *Trachurus japonicus* belonging to the subfamily Trachinotinae indicated in pink (75% value of bootstrap); **Group 3** included two species of *Seriolina nigrofasciata* and *Seriola quinqueradiata* belonging to subfamily Naucratinae indicated in yellow (89% value of bootstrap); **Group 4** indicated in red line included 12 species (*Alectis indicus, Alepes kleinii, Alepes vari, Atule mate, Selar crumenophthalmus, Trachurus japonicus, Decapterus maruadsi, Parastromateus niger, Carangoides malabaricus, Carangoides ferdau, Atropus atropos, Megalaspis cordyla, Uraspis uraspis*) of Caranginae subfamily with the lowest bootstrap value (39%).

The phylogenetic relationship of Carangidae has also been published by many authors. In which, Gushiken (1988), Kijima et al., (1986), SmithVaniz (1984) and Reed (2002) [28–30, 37], examined the phylogenetic relationships in the Carangidae family in previous studies, Smith -Vaniz (1984) provided more information about the close relationship between the two subfamilies (Caranginae and Naucratiniae). Kijima et al., (1986) proposed that Scomberoidiniae and Trachinotiniae were closely related to the subfamily Naucratiniae which was a part of subfamily of Caranginae

latter. In particular, this result was similar with previous studies as Gushiken (1988) and Reed (2001), thereby the authors have proposed a phylogenetic tree consisting of two main lineages, in which one lineage included Caranginae and Naucratiniae, and remaining lineage consisted of Scomberoidiniae and Trachinotiniae. The phylogenetic relationships in the genera of the Caranginae subfamily were more complex, the previous studies (Gushiken (1988); Kijima et al., (1986); Smith-Vaniz, (1984)) were agreed on the number, relationship and the each genus from each lineage. In present study, the Caranginae subfamily consisting of 3 branches was similar to the previous study of Reed (2001), in which, (A) branch included Selaroides; (B) branch included Alectis, Uraspis, Megalaspis; (C) branch included Atropus, Carangoides, Parastromateus, Alepes, Decapterus, Trachurus, Selar, Atute (fig. 3b), only A branch was well supported by bootstrap value with 99%. Gushiken (1988) was the first person to point out that the two Carangoides and Alectis may be sub-branches in the Caranginae subfamily. But in present study, the number of specimens was not enough to check all the taxa carefully and to analyze ancestors and later generations of the Caranginae subfamily. Currently, the database in this study on Caranginae subfamily could supported the single-branch hypothesis of some genera such as Decapterus, Trachurus.

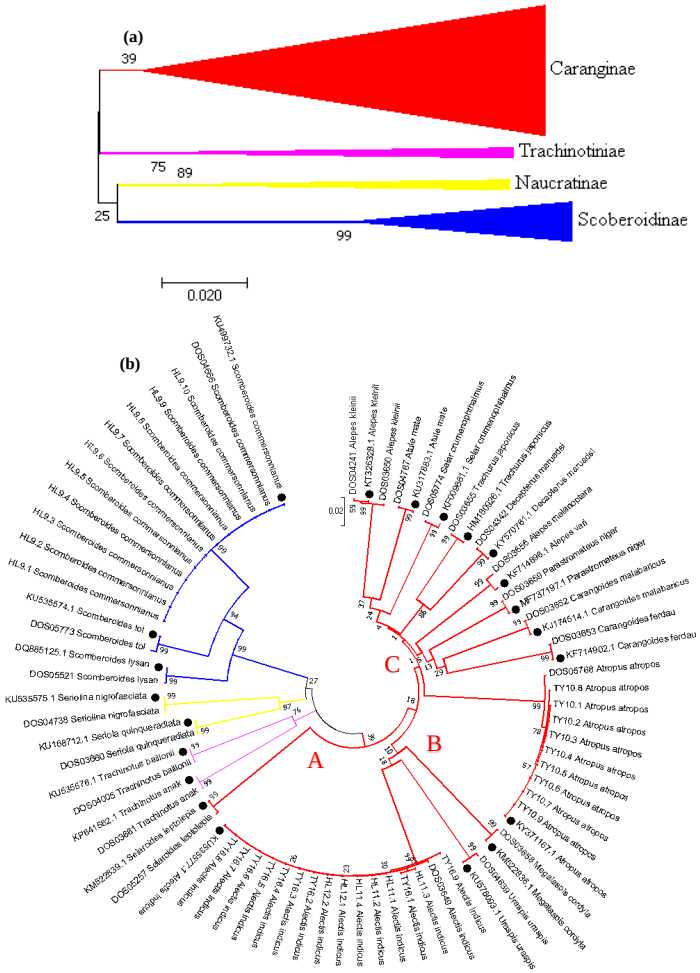


Fig. 3. The phylogenetic tree was based on the COI gene with the GTR + G + I model following the Neighbor Joining method, with a bootstrap value of 1000 times (*4 subfamilies indicated by color; species on GenBank denoted by black dots at branches*)

Efficiency between two classification methods

The morphological classification has the advantages as fast and economic method, many references, no requirement on modern equipment and expensive chemicals but it requires much experience from experts. Specially, the traditional morphological method for species identification is 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 and shows the effectiveness, high accuracy and resolves taxonomic ambiguities in many cases. In present study, the DNA barcoding method (COI gene) identified 21 species (16 genera) while the morphological method only identified 18 species (16 genera) from 56 specimens (table 1). In particular, some specimens which could not be identified or was identified incorrectly according to the morphological method has been identified to species and revised by DNA barcoding method (table 1). In addition, the effectiveness of the DNA barcoding method has also been shown in many previous studies [31–35, 41]. Thus, the previous studies 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 Carangidae.

CONCLUSION

56 specimens of Carangidae collected from three coastal areas (Northern, Central and Southern) in Vietnam were identified to 21 species belonging to 16 genera by the COI barcoding method while 18 species (16 genera) were determined by morphological method. *Seriola quinqueradiata* and *Trachinotus anak* were new records for Carangidae in Vietnam. The DNA barcodes of COI gene of 21 species in Carangidae in present study could be used as a basis for comparing, estimating phylogenetic diversity and traceability of their products as well as other studies of Carangidae in conservation, management and utilization of fisheries resources. Once again, the results showed the high potentiality in using COI barcode to identify Carangidae fish in Vietnam.

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