Journal of Sustainability Science and Management
Volume 15 *Number* 4, June 2020: 34-44
eISSN: 2672-7226

CYTOCHROME OXIDASE I GENE REVEALS POTENTIAL CRYPTIC DIVERSITY OF DOUBLEWHIP THREADFIN BREAM, *Nemipterus nematophorus* (BLEEKER, 1854) IN PENINSULAR MALAYSIA

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Submitted: 27 August 2019 *Accepted: 16 April 2020* http://doi.org/10.46754/jssm.2020.06.004

Abstract: Genetic variations and differences among three *Nemipterus nematophorus* populations in Peninsular Malaysia were evaluated based on partial sequence of the mitochondrial Cytochrome Oxidase I gene (654bp). Among the 30 individuals sampled, 12 putative haplotypes were detected, and 58 % (7) of the haplotypes were unique with a high level of haplotype diversity (H= 0.802) and low nucleotide diversity ($\boldsymbol{\pi}$ = 0.0114). The constructed neighbour joining (NJ) phylogenetic tree based on haplotypes showed two clusters with specimens from Kelantan and Melaka grouped in the same cluster, while Terengganu samples formed an isolated cluster. These two clusters were separated with maximum 2.9 % nucleotide divergence. Analysis of Molecular Variance (AMOVA) revealed a high level of F_{ST} value (F_{ST}= 0.16; p=0). Pairwise F_{ST} value showed significant differences between the Kelantan and Melaka populations from the one in Terengganu. The Terengganu population, although morphologically identical with specimens from Kelantan and Melaka, consisted of a genetically discrete taxon. This study revealed that there is potential cryptic diversity of *N. nematophorus* in Peninsular Malaysia. These findings are important to provide a scientific framework for sustainable management strategies and conservation of commercially important fishery resources in the region.

Keywords: COI, cryptic diversity, *Nemipterus nematophorus*, Peninsular Malaysia.

Introduction

Malaysia is one of the biodiversity hotspots in Southeast Asia (Lim *et al.*, 2016). Its inland and coastal waters are known as rich fish breeding habitats, and about 82 % of Malaysia's national fish harvest comes from inshore fisheries (Lim *et al.*, 2016). The studied species, the doublewhip threadfin bream or *Nemipterus nematophorus* (locally known as *ikan kerisi dwifilamen*), is a bottom-living coastal fish confined to tropical and sub-tropical waters of the Indo-West Pacific region (Hung *et al.*, 2017).This species has been receiving increasing attention because of its commercial importance, specifically in the manufacture of surimi and surimi-based products (Santos & Ng, 1993). *Nemipterus nematophorus* is available throughout the year and very popular with Malaysian consumers (Imtiaz *et al.*, 2016). Threadfin breams are an important demersal fishery resource along the Malaysian coast. They are mainly exploited by small commercial trawlers in depths up to 50 m. The existence of rich resources of threadfin breams on the continental shelf beyond 50 m depth, especially in the 75 to100 m belt along different parts of the shelf, often form 75 % of the trawl catch (Joseph, 2000). However, threadfins bream catches are rarely reported because they are hard to identify (Pawar *et al.*, 2011), and misidentification is a common problem at fish jetties and markets (Imtiaz *et al.*, 2016).

Commercially important marine species may be particularly at risk in the loss of genetic diversity because population boundaries are often difficult to identify, migration patterns are not well described, and harvest may preferentially target specific population segments. Population genetic analysis is the best method to evaluate genetic divergence and plays vital role in getting information about the conservation

genetics of a species (Xu *et al.*, 2014)2014. It is also used to improve knowledge of the stock structure and can provide guidelines to optimize these practices and thus, conserve the genetic structure of *N. nematophorus*.

Molecular markers have been widely used in population genetics studies to identify fish stock structure and to infer population history (Sun *et al.*, 2012; Habib & Sulaiman, 2016). DNA markers are becoming more popular as well in obtaining gene flow information, allele frequencies and other parameters, which are crucial in population biology (Habib & Sulaiman, 2016). Mitochondrial DNA (mtDNA) markers are more sensitive to demographic events that may affect genetic variation, such as reduction in population size and geographic isolation (Durand *et al.*, 2005). Therefore, mtDNA has remained the marker of choice in many population, phylogenetic, phylogeographic and biogeographic studies (Hurst & Jiggins, 2005). It is also widely used to investigate genetic variation between populations, population history, origin, migration or evolution (Lee *et al.*, 2011). In the mitochondrial genome, the Cytochrome Oxidase-I gene (COI) is a recognized marker for genetic diversity detection and population genetic structures of marine fishes (Habib *et al.*, 2011; Sun *et al.*, 2012; Xu *et al.*, 2012; Xu *et al.*, 2014; Mat Jaafar *et al*., 2019). COI is also appropriate for species identification, particularly the cryptic species (Imtiaz *et al.*, 2016), and this has been used for both marine (Zhang, 2011; Mat Jaafar *et al*., 2012; Wang *et al.,* 2012; Chang *et al*., 2017; Bakar *et al*., 2018) and freshwater fishes (Hubert *et al.,* 2008; Barman *et al*., 2018; Rahman *et al*., 2019). The present study aimed to understand the population genetic structure and potential cryptic diversity of *N. nematophorus* along the coasts of Peninsular Malaysia for developing a sustainable management plan for this commercially important fishery resource based on COI gene as a marker.

Materials and methods

Samples collection

Samples of *N.nematophorus* were collected from fish markets and landing sites within Peninsular Malaysia (Melaka, Terengganu and Kelantan) as shown in Figure 1. Specimens of N. *nematophorus* were identified using fish identification books (Ambak *et al*., 2010; Yoshida *et al*., 2013; Lim *et al*., 2018). This species could be differentiated from other *Nemipterus* species by its elongated first spine of dorsal fin and yellow filamentous upper lobe

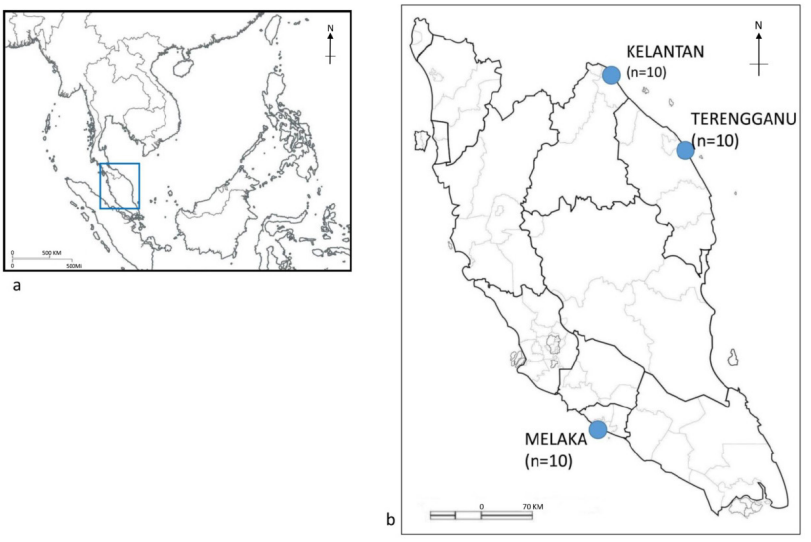

Figure 1: Sampling sites of *N. nematophorus* in (a) Peninsular Malaysia (marked with blue box in the map of Southeast Asia) and (b) sampling locations

of the caudal fin (Yoshida *et al*., 2013). A total of 30 specimens were collected, with 10 individuals from each location. The right pectoral fin of the samples was preserved in 95 % ethanol and kept at –4°C until further analyses.

DNA extraction and PCR amplification

Total genomic DNA from each specimen was extracted using salt extraction methods according to Miller *et al.* (1988). The concentration and purity of the extracted DNA stock were determined using the Analytik Jena ScanDrop nano-volume spectrophotometer (Analytik Jena AG, Jena, Germany) before being stored in 1.5 µL microcentrifuge tubes at -20 ^{0}C. PCR amplification of mtDNA COI was performed in 25 µL solutions comprising 2.0 µL of DNA extract, 0.3 µL of forward and reverse primers (10mM), 2.5 µL of 10X PCR buffer, 2.0 µL of $MgCl_2$ (50 mM), 0.25 µL of dNTP mix (10 mM/µL), 0.20 µL of Taq polymerase (5U/µL) and 17.45 µL of pure distilled water. The sequences of the primers used were (FishF1) 5'TCAACCAACCACAAAGACATTG GCAC3' and (FishR1) 5'TAGACTTCTGGGTG GCCAAAGAATCA3'(Ward *et al*., 2005).

The PCR reaction was performed using an Analytik Jena SpeedCycler (Analytik Jena AG, Jena, Germany) with the following program: three minutes (min) at 94 °C for initial denaturation, followed by 40 cycles of denaturing at 94 °C for 45 seconds, annealing at 53 °C for 45 seconds, and extension at 72 °C for 90 seconds, with a final five-minute extension at 72 °C before the process was terminated at 4 °C (Ward *et al.*, 2005).The PCR products were visualized on a 1.2% (w/v) agarose gel stained with SyBr Safe (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were sent for sequencing (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) using forward primer only.

Sequence editing and alignment

All COI sequences were edited and aligned using ClustalW implemented in MEGA 6.0 (Tamura *et al.*, 2013). The sequences were translated into amino acid sequences to ensure accurate alignment and no detection of numts. Confirmation of species identification were done by comparing all COI sequences with GenBank (http://blast.ncbi.nlm.nih.gov) (Benson *et al*., 2013) and BOLD system databases (www.boldsystems.org) (Ratnasingham & Hebert, 2007) to avoid misidentification of specimens. Similarity thresholds of 99% were used to assign specimens to species level. All sequences were deposited into GenBank under the accession numbers MN808440 – MN808513.

Data analyses

The complete aligned dataset was analysed for genetic diversity, including number of haplotype, haplotype diversity (Hd) and nucleotide diversity (π) using DnaSP6.0 (Rozas *et al*., 2017).

The Arlequin software package version 3.5.1.2 (Excoffier & Lischer, 2010) was used to perform a hierarchical analysis of molecular variance (AMOVA) to examine population structure of *N. nematophorus* within the east and west coast of Peninsular Malaysia. The pairwise F_{ST} was generated to calculate relative genetic differentiation between populations based on Tamura-Nei distance method (Tamura & Nei, 1993), and statistically significant pairwise comparisons were tested with 10,000 permutations (Xu *et al.*, 2014)2014.

Neighbor-Joining (NJ) tree was constructed using MEGA6.0 and *Caranx sexfasciatus* (HQ560947) was included as out-group. The Kimura Two-Parameter (K2P) evolutionary distance (Kimura, 1980) was used with the NJ method and the confidence levels at each node were assessed by 10,000 bootstrap replications. A Maximum Likelihood (General Time Reversible model, GTR) (Nei & Kumar, 2000) approach of the mitochondrial loci was conducted by determining the highest likelihood tree bootstrapped 10,000 times using RAxML 7.2.8 (Stamatakis *et al.*, 2008). A network of all haplotypes was constructed by median joining calculation in Network 10.0.0.0 (Bandelt*et al*., 1999) to view the phylogeographic relationship

among haplotypes.

Results and Discussion

Species identification

All 30 COI sequences in this study had been compared with sequences in GenBank with 99.7% similarity to *N. nematophorus* (MH235681, KY362838). Therefore, this confirmed that all specimens collected were correctly identified as *N. nematophorus*.

Genetic diversity

A total of 30 samples were assayed from three populations of *N. nematophorus* for the 654-base pair (bp) COI sequence. No stop codons were observed, confirming that all amplified sequences constituted functional mitochondrial COI sequences. There were 21 polymorphic nucleotide sites, of which 16 were parsimony informative. Twelve unique haplotypes were identified with haplotypic diversity ranging from 0.511 to 0.889 (Table 1). Two haplotypes were recovered more than once. These two haplotypes (H_7 and H_10) were shared by samples from Kelantan and Melaka. While the other 10 haplotypes being singletons. The Melaka population had the lowest haplotype (0.511) and nucleotide (0.00087) diversities, while Terengganu samples had the highest values at 0.889 and 0.00284, respectively. Although all populations showed high haplotype diversity, low nucleotide diversity was also observed, ranging from 0.00087 to 0.00284. This indicated that tehre were small genetic differences only between haplotypes.

The pairwise distance value between the three *N. nematophorus* populations ranged from 0.1 % to 2.5 %. The Kelantan and Melaka populations were the closest at 0.1 % nucleotide divergence, while Terengganu population was the most diverged with average pairwise nucleotide divergence of 2.45 % (Table 2).

The analysis of mtDNA COI sequences of *N. nematophorus* from three distant populations identified 12 distinct haplotypes. The high level of haplotype diversity (h=0.802) and low nucleotide diversity were observed. Large and stable population size of marine fishes might lead to higher level of genetic diversity (Avise, 1998). In Peninsular Malaysia, *N. nematophorus* was one of the most extensively distributed and commonly landed fish species (Lim *et al.,* 2016). So, its large population might support

Table 1: Distribution of haplotype frequencies in COI gene of *Nemipterus nematophorus*

Haplotype	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**n**	**Hd**	**π**
Localities															
Terengganu	2	1	2	3	1	1							10	0.889	0.00284
Kelantan							6	1	1	1	1		10	0.667	0.00125
Melaka							7			2		1	10	0.511	0.00087
Total														0.802	0.0114

Symbols equal: n, sample size; Hd, haplotype diversity; π, nucleotide diversity

Table 2: Pairwise nucleotide distance (below diagonal); pairwise F_{ST} values (above diagonal) of COI gene between *N. nematophorus* populations

Populations	**Terengganu**	**Kelantan**	**Melaka**
Terengganu		0.2*	0.3*
Kelantan	0.025		-0.05159
Melaka	0.024	0.001	

Symbol * showed significant pairwise *F*ST values (P<0.05)

the high levels of haplotype diversity that was observed in this study. According to Grant and Bowen (1998), high haplotype diversity and low nucleotide diversity could also occur with the following two reasons: (i) a population experiencing bottleneck or about to go extinct, and (ii) allele losses followed by quick population growth from a small population, assuming that there was enough time for retrieval of haplotype variation through mutation.

Similar patterns had been reported for the Japanese threadfin bream (*Nemipterus japonicus*) (Lim *et al.*, 2016), miiuy croaker (*Miichthys miiuy*) (Cheng *et al*., 2011; Xu *et al.*, 2014)2014, crevalle jack (*Caranx hippos*) (Hernandez *et al*., 2018), white croaker (*Pennahia argentata*) (Han *et al.*, 2008) and Crimson snapper (*Lutjanus erythropterus*) (Zhang *et al*., 2006). The population genetic study of *N. japonicus* also found low nucleotide diversity. The study explained that lower nucleotide diversity might occur due to the shallow shelf of Peninsular Malaysian coastal waters that had been recolonized by a reduced population of *N. japonicus* since the end of the last glacial maximum (Lim *et al.*, 2016). For future adaptation, genetic diversity within and between populations provided a potential genetic resource, and could be critical for the suitability of a population (Hurt & Hedrick, 2004). The gene flow across large spatial scales within Malaysia was indicated by the presence of shared haplotypes of samples at two distant locations, which were Kelantan and Melaka.

Phylogenetic analysis, population genetic structure and gene flow

The NJ tree revealed two clusters of *N. nematophorus* (Figure 2). Mean K2P distance within species was 1.2 %, with a maximum of 2.9 % nucleotide divergence. Cluster I consisted of populations from Melaka and Kelantan supported by 98 % bootstrap value. While Cluster II comprised all individuals from Terengganu with 89 % bootstrap value. The Maximum Likelihood (ML) tree also showed the same pattern (not shown).

For AMOVA, the variance among populations, among populations within groups and among groups relative to the total variance were F_{ST}= 0.160, p=0; F_{SC}=0.251, p=0.001; F_{CT}=-0.123, p=0.667, respectively (Table 3). The result indicated a significant variation among the population and population within group. The pairwise F_{ST} values showed there were significant differentiations between Kelantan and Melaka populations with the Terengganu population. However, there was no significant difference detected between Kelantan and Melaka populations (Table 2). The median joining network based on nucleotide distance among haplotypes indicated two clades (Clade A & B) (Figure 3), which was consistent with NJ and ML phylogenetic trees. Clade A consisted of haplotypes from Kelantan and Melaka and was separated from Clade B by numerous mutational steps. The Terengganu haplotypes included in Clade B and related to each other except for Hap06, which was isolated but still within Clade B. One intermediate hypothetical haplotypes (mv1) was detected, probable of haplotype extinction or un-sampled specimen. Seven of the haplotypes (58%) are singletons, indicating a recent population expansion.

Table 3: Analysis of molecular variance (AMOVA) of *N. nematophorus* populations

Source of variation	Percentage of variation	F-statistic	P-value
Among group (F_{CT})	6.84	-0.12195	0.66862
Among population within group (F_{SC})	-0.12	0.25121	0.00098
Withinpopulations (F_{ST})	93.04	0.160	0

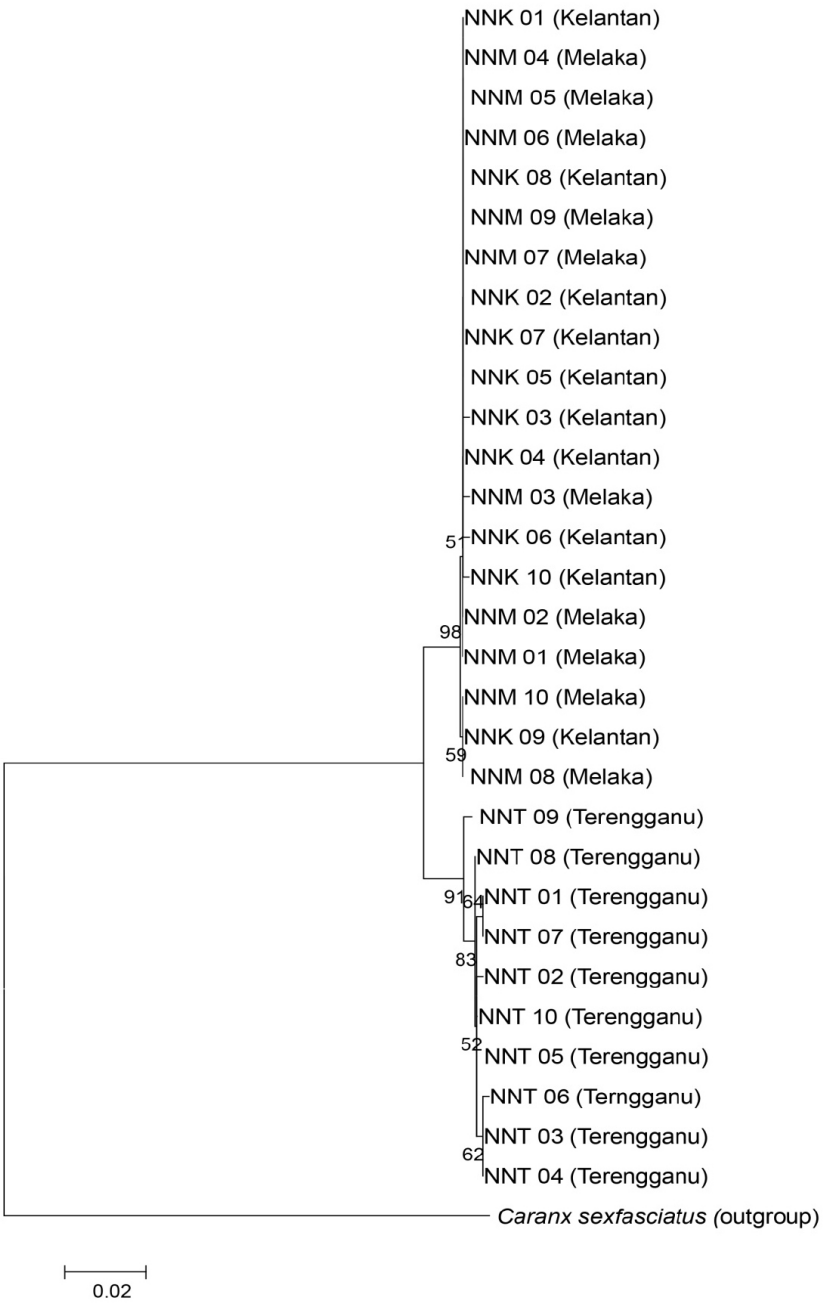

Figure 2: Neighbour joining tree (NJ) of *N. nematophorus* using the Cytochrome oxidase subunit I sequences. Only bootstrap values greater than 50 are shown

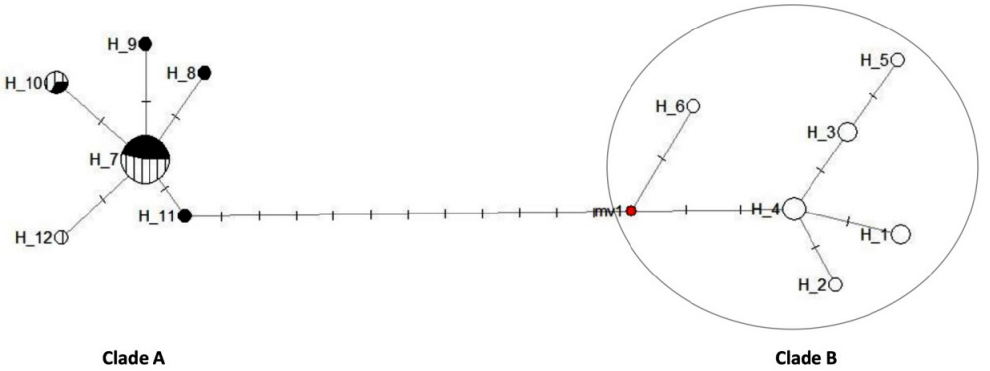

Figure 3: A minimum spanning network of COI gene haplotypes from *N. nematophorus* populations. Solid black represents haplotypes from Kelantan, solid white from Terengganu, vertical from Melaka. Crossbars on connecting line represents step of mutation between the haplotypes, mv is missing haplotypes linking the clades. Size of the circle is proportional to number of individuals

Marine fishes typically showed low levels of genetic differentiation in different geographical regions despite their high dispersal of potential planktonic egg, larval or adult history, along with the lack of physical barriers on movement between ocean basins or nearby continental margins (Avise *et al.*, 1987; Rivera *et al.*, 2004; Xu *et al.*, 2014). Evidence from this study also showed that levels of genetic differentiation using F_{ST} values inferred from COI marker were low in *N. nematophorus*, indicating extensive gene flow among distant populations. There were no barriers to gene flow from Melaka (west coast) to Kelantan (east coast), as specimens from these two localities were observed to share the same haplotype.

However, to confirm the occurrence of a single homogenous population of *N. nematophorus* within Malaysia waters, additional samples from other localities should be included in future studies. Similar results were found in *N. japonicus,* which was conducted in Peninsular Malaysia waters by Lim *et al*., (2016). They found that *N. japonicus* populations off the peninsula were panmictic, except a population in Kuala Besar, which was found to be a genetically discrete taxon from the rest. Mat Jaafar *et al*. (2019) also found no significant population subdivisions among bigeye scads (*Selar crumenophthalmus*) in Malaysian waters. In contrast, the other two Carangid species, the yellowtail scad (*Atule mate)* and yellowstripe scad (*Selaroides leptolepis*) showed significant differentiation among populations within this region. However, no geographic structuring was observed.

Although the current study indicated low genetic differentiation between *N. nematophorus* populations, a few studies have shown that population subdivisions did occur in marine fishes even across small spatial scales (Kakioka *et al*., 2018; Mat Jaafar *et al*., 2019). Some of the marine fishes consisted of discrete lineages within the same species due to the presence of geographic barriers and vicariance events in marine environment (Rocha *et al*., 2007; Drew & Barber, 2009; Leray *et al*., 2010; Kakioka *et al*., 2018). In addition, genetic differentiation in some marine fishes had also been linked to circulation patterns and water exchanges between seas or oceans (Rohfritsch & Borsa, 2005).

Potential cryptic species

Many species previously known to disperse widespread in marine environment could now be traced back to discrete lineages through the application of molecular methods (Lim *et al*., 2016; Imtiaz*et al*., 2016; Mila *et al*., 2017; Bakar *et al*., 2018; Kakioka *et al*., 2018; Mat Jaafar *et al*., 2019). It had also become

clear that the occurrences of cryptic species were quite common in marine environments (Lindstrom *et al*., 2012; Martinez-Takeshita *et al*., 2015; Lim *et al*., 2016; Bakar *et al*., 2018). This study observed two discreet lineages of *N. nematophorus* in Peninsular Malaysia with a mean K2P distance within species of 1.2 % and a maximum of 2.9 % nucleotide divergence. Cluster I consisted of samples from Melaka and Kelantan, while Cluster II comprised specimens from Terengganu only. However, no geographic pattern was apparent as Kelantan and Terengganu were in the northeast of Peninsular Malaysia, while Melaka was in the southwest. The genetic divergence among the three populations ranged from 0.1 % to 2.5 % with the largest differences seen between Kelantan and Melaka populations with Terengganu ones. The Terengganu population, although morphologically identical with specimens from Kelantan and Melaka, was composed of a genetically discrete taxon. This level of genetic divergence was consistent with the previous study of *N. japonicus* along the coasts of Peninsular Malaysia (Lim *et al.*, 2016), which also found distinct cryptic species. Bakar *et al*., (2018) uncovered two different lineages among bigeye snappers (*Lutjanus lutjanus*) in Peninsular Malaysia. One of the lineages could represent an unrecognized or cryptic species. Mat Jaafar *et al*. (2019) also confirmed the existence of cryptic species among yellowtail scads in Malaysian waters. However, further examination on morphological characteristics would be required to confirm the new taxons.

Conclusion

Present study revealed the existence of potential cryptic diversity of *N. nematophorus* in Malaysian waters. The Terengganu population was very likely a different taxon from Kelantan and Melaka, but further investigations into its genetic characteristics, morphometrics, meristics and osteological characteristics were required to confirm and declare such new taxons. The evidence also showed that subdivisions exist in *N. nematophorus* populations in the coasts of Peninsular Malaysia and should be considered as different management units for effective conservation and management purposes. However, we only examined a single gene from mitochondrial DNA. The use of multiple genes, including nuclear markers, should be considered to increase the resolving power of genetic studies and for a better understanding on the population structure of *N. nematophorus* within this region.

Acknowledgements

The authors like to thank the Fisheries Department of Malaysia for the opportunity to participate in the National Demersal Trawl Survey expedition in 2016. The authors also thank Universiti Malaysia Terengganu for providing the logistics and financial support.

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