BIODIVERSITAS
Volume 17, Number 2, October 2016
Pages: 571-577

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d170226

Molecular identification of commercially important species of *Nemipterus* (Perciformes: Nemipteridae) in surrounding seas of Malaysia

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Manuscript received: 17 May 2016. Revision accepted: 19 July 2016.

Abstract. *Imtiaz A, Duong TY, Nor SAM, Naim DM. 2016. Molecular identification of commercially important species of Nemipterus (Perciformes: Nemipteridae) in surrounding seas of Malaysia. Biodiversitas 17: 571-577.* The genus *Nemipterus* is a group of coral fishes which are morphologically diversified in coloration and distribution pattern. In this study, the mitochondrial Cytochrome c oxidase-I (COI) gene was analyzed for genetic identification of 127 samples of genus *Nemipterus* from Malaysia waters. Sequence analysis of COI based data clearly distributed ten putative species into four distinct clusters clades. Intra-specific genetic distance values of 2.7%, 3.4% were observed in *N. japonicus* and *N. nemurus* which require more detailed analysis of the taxonomic status of some of the individuals attributing to slightly atypical values. Neighbor joining (NJ) tree shows a low genetic structuring in *N. japonicus*. Populations from Indian Ocean and South China Sea are isolated from each other but both share genetic structure with the population from the Straits of Malacca, suggesting the possibility of the latter acting as a barrier to the movement of this species between the two neighboring seas.

Keywords: Coral fishes, genetic structure, intra-specific, Cytochrome c oxidase-I, Malaysia, *Nemipterus*, South China Sea

INTRODUCTION

Integrated land of Malaysia is a combination of the southeastern tip of the Asian mainland (Peninsular Malaysia) and the states of Sabah and Sarawak in the western part of Borneo Island. Peninsular Malaysia faces the Strait of Malacca (extension of the Indian Ocean) towards the west and the South China Sea towards the east. Sarawak faces the South China Sea while Sabah faces the South China Sea, Sulu Sea and Celebes Sea. Malaysia has the potential to be one of the leading fishing nations in the Southeast Asian region, and is poised to step into a new era of development in the marine fishing industry. However, the ill-effects from past management programs have created many problems and conflicts for Malaysia in managing her own resources to achieve optimum utilization. One of the main issues is conservation planning. In the early stages of development, the country failed to take conservation measures into serious consideration, which resulted in inshore waters being over-exploited.

One of the impacted fish genus is *Nemipterus* from the family Nemipteridae. They - are fished throughout the year and are very popular with Malaysian consumers. According to Abu Talib (2003) and Mohd Taupek (1996), decline of *Nemipterus* has been occurring for some time as observed through trend landing analysis. Family Nemipteridae worldwide consists of five genera comprising 67 species. Within genus *Nemipterus* 25 species are present worldwide with 19 species documented in Malaysia (Edward 1992; Russell 1990). Members under this genus are well known for their delicious taste and commercial importance. They are commonly called threadfin breams and can be identified by their pinkish body coloration with variable yellow streaks on the whole abdominal length and fins. Threadfin breams are demersal fish and are residents of the Indo West Pacific biogeographical region (Russell 1990). They feed mainly on crustaceans. They are small to medium sized fish and can be easily caught through trawling. Although the taxonomy of the genus has been largely defined and resolved on the basis of morphological characters (Russell 1986, 1990, 1993), a number of taxonomic issues still remain. By relying solely on external morphology, subtle differences may not be detected and could lead to misidentification, for example *N. randalli* was misidentified as *N. japonicus* (Lelli 2008).

Misidentification is a common occurrence at fish trading places such as the market and landing sites. Precise identification of fish is important for the satisfaction, value for money and well-being, in the case of allergies, to the consumers. Often a species may be known by alternative vernacular names and synonyms which could lead to much confusion. Kannuchamy (2015) detected mislabeling of 22% of frozen seafood prevailing in Indian markets. Changizi (2013) revealed incorrect labelling of the Narrow-barred Spanish mackerel samples in Iranian fish products. Data on the genetic identity of populations/species are

essential when designing programs for the conservation and management of fish. DNA barcoding is a molecular technique that is now widely accepted as a tool for taxonomic identification of various organisms including fish species. It involves the amplification of a specific segment of the mitochondrial DNA which is sufficiently variable to distinguish between species but conserved within species. In fish, the DNA barcoding region is a 650 base pair fragment of the mitochondrial cytochrome oxidase I (COI) gene and has been widely utilized to authenticate for species identification, particularly in the case of cryptic species and ambiguous morphological characteristics (Hubert et al. 2008, Aquilino et al. 2011, Sanciangco et al. 2011). The COI gene group is considered to be efficient for species level taxonomic identification because it consists of only protein encoding genes (Brown 1979) and have slow evolutionary rate (Saccone et al. 1999) which can help in elucidating evolutionary divergences (Lynch and Jarrell 1993). DNA barcoding has proven to be very successful for systematic investigation of a wide variety of marine fish taxa (Ward et al. 2005, Lakra et al. 2010, Wang et al. 2012). In South East Asia DNA barcoding has not been applied widely although there are few reports on marine organisms i.e. Suzanna et al. (2011) used DNA barcoding to discriminate oysters species. Jaafar et al. (2012) conducted a comprehensive DNA barcoding study on family Carangidae.

Thus, the current research aims to genetically identify the species of genus *Nemipterus* in the surrounding seas of Malaysia. To the fisheries managers, our study contributes invaluable complementary data for biodiversity assessment and recognition of cryptic species for sustainable fisheries of this genus.

MATERIALS AND METHODS

Sample collection

A total of 127 samples of genus *Nemipterus* were obtained from Malaysian waters consisting of Straits of Malacca, South China Sea, Sulu Sea and Celebes Sea (Figure 1). All samples were morphologically identified into nine species (*N. japonicus, N. hexodon, N. tambuloides, N. peronii, N. thosaporni, N. nematophorus, N. bipunctatus, N. marginatus,* and *N. furcosus*). In addition, we included fifteen specimens belonged to four species (*N. hexodon, N. japonicus, N. furcosus, N. tambuloides*) from Vietnam and seven specimens of one species (*N. japonicus*) from Pakistan as regional conspecific comparison with our samples (Table 1) and a specimen identified as *Scolopsis vosmeri* as an out-group. All samples were photographed and have been kept as voucher specimens at Zoological Museum in Biodiversity Centre, Universiti Sains Malaysia after preservation in 95% alcohol. The morphological identification was based on FAO catalog of genus *Nemipterus* (Russell 1990).

DNA Isolation

Total genomic DNA was extracted by salt extraction (Animal Genomics Laboratory, Liverpool University, United Kingdom, (2001) in the presence of proteinase K (Nacalai Tesque, Japan) with some modification on the amount of TNES-Urea used to improve the yield and quality of the extracted DNA. The DNA pellet obtained was then eluted with deionized water. Quality and quantity of isolated DNA were measured using spectrophotometer (Quawell, Korea) and stored at-20°C until further use.

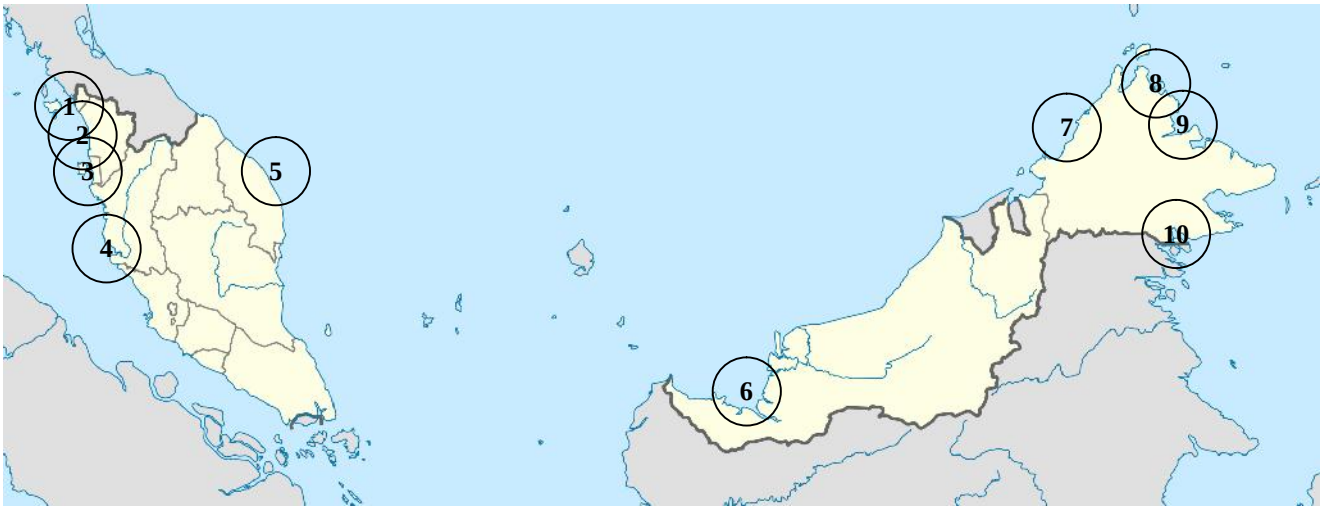

Figure 1. Sampling locations of species collected from Malaysian waters (Strait of Malacca, South China Sea and Sulu Sea, Celebes Sea). Note: 1. Kuala Kedah, Kedah (KK), 2. Kuala Perlis, Perlis (KP), 3. Batu Lanchang, Pemang (BL), 4. Lumut, Perak (ML), 5. Kuala Terengganu,Terengganu (TBK), 6. Kuching, Sabah (KCH), 7. Kota Kinabalu, Sabah (KTK), 8. Kudat, Sabah (KD), 9. Tawau, Sabah (TW), 10. Sandakan, Sabah (SDK), 11. Vietnam (NRC,VCM,VBL), 12.Pakistan (Pak)

Table 1. Total number of *Nemipterus* species and their respective locations

Species name	Locations of sampling												Total no. of Samples
	1	2	3	4	5	6	7	8	9	10	11	12	
N. hexodon	2	2	0	0	0	0	1	0	1	1	2	0	9
N. japonicas	6	6	6	8	0	0	1	1	4	5	5	7	47
N. tambuloides	0	0	0	0	0	0	0	2	0	0	2	0	4
N. bipunctatus	0	3	0	9	0	0	0	0	0	0	0	0	12
N. furcosus	0	0	0	9	0	0	0	0	6	4	6	0	25
N. nematophorus	0	1	0	0	0	0	1	0	0	0	0	0	2
N. marginatus	0	2	0	0	3	0	2	1	1	1	0	0	10
N. thosaporni	0	0	0	0	0	0	0	1	5	5	0	0	11
N. peronii	0	0	0	0	0	0	1	0	2	1	0	0	4
N. nemurus	0	0	0	0	0	1	1	1	0	0	0	0	3

Note: 1. Kuala Kedah, Kedah (KK), 2. Kuala Perlis, Perlis (KP), 3. Batu Lanchang, Pemang (BL), 4. Lumut, Perak (ML), 5. Kuala Terengganu, Terengganu (TBK), 6. Kuching, Sabah (KCH), 7. Kota Kinabalu, Sabah (KTK), 8. Kudat, Sabah (KD), 9. Tawau, Sabah (TW), 10. Sandakan, Sabah (SDK), 11. Vietnam (NRC,VCM,VBL), 12.Pakistan (Pak)

Table 2. Sequences Accessed from Bold and Genbank with species names and areas of Collection sites

BOLD/Genbank Accession numbers	Species name	Collection site
ANGEN128-15	*N. japonicus*	Indian Ocean
ANGEN16115	*N. japonicus*	Indian Ocean
LGEN09314	*N. japonicus*	Indian Ocean
EF609556	*N. japonicus*	Indian Ocean
EF609553	*N. japonicus*	Indian Ocean
FJ347947	*N. japonicus*	Indian Ocean
JQ691509	*N. japonicus*	South China Sea
EU871686	*N. japonicus*	South China Sea
JF493971	*N. japonicus*	South China Sea
EU871687	*N. japonicus*	South China Sea
HQ676778	*N. marginatus*	Indian Ocean
JQ681506	*N. marginatus*	South China Sea
HQ423413	*N. bipunctatus*	Indian Ocean
JQ350137	*N. bipunctatus*	Indian Ocean
EF609414	*N. hexodon*	Pacific Ocean*
FJ237848	*N. virgatus*	Unknown
JN992286	*N. nematophorus*	Indian Ocean
JQ681467	*N. bathybius*	South China Sea
JN992287	*N. zysron*	unknown
EF609557	*N. mesoprion*	Indian Ocean
JX866609	*N. mesoprion*	Indian Ocean
EF609561	*N. mesoprion*	Indian Ocean
EF609415	*N. peronii*	Pacific Ocean*
EF609413	*N. furcosus*	Pacific Ocean*
JQ681525	*N. furcosus*	South China Sea
JN992288	*N. japonicus*	Indian Ocean

Note: *Pacific Ocean = Near Australia

Amplification and sequencing

The COI gene was amplified in a 50 µL volume solution with 5 µL of 10X PCR buffer, 3.5 µL of 50mM MgCl2, 2 µL of 0.05mM dNTP, 1 µL of 0.01mM each primer, 0.5 units of *iTaq* plus DNA polymerase and 50-100ng of genomic DNA template. The primers used for the amplification of the COI gene were FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and FishR1 5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward et al. 2005). The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 45 s at 94°C, 45 s at 50°C and 1 min at 72°C and a final extension of 10 min at 72°C. The PCR products were visualized on 2 % agarose gels, and only intense and sharp bands were selected for purification as recommended in Intron Purification Kit (Intron, South Korea). The cleaned PCR products were sequenced by a service provider, 1st BASE Sequencing Service Sdn. Bhd.

Data analysis

The obtained sequences (Tamura et al. 2007) were edited by combining the forward and reverse sequences and ambiguous sites were deleted. Multiple alignments were then performed and Kimura 2-Parameter was selected to infer genetic variability as estimated by nucleotide diversity, haplotype diversity and pairwise genetic distance among haplotypes. Neighbors-joining (NJ) tree was then constructed using 10, 000 bootstrap replications. Twenty-six sequences (ANGEN128-15, ANGEN16115, LGEN09314, EF609556, EF609553, FJ347947, JQ691509, EU871686, JF493971, EU871687, HQ676778, JQ681506, HQ423413, JQ350137, EF609414, FJ237848, JN992286, JQ681467, JN992287, EF609557, JX866609, EF609561, EF609415, EF609413, JQ681525, and JN992288) were retrieved from GenBank and BOLD systems for comparison (Table 2). All analyses were conducted in MEGA 6.06 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Sample collection

A total of 127 samples of genus *Nemipterus* were obtained from Malaysian waters consisting of Straits of Malacca, South China Sea, Sulu Sea and Celebes Sea. All samples were morphologically identified into nine species (*N. japonicus*, *N. hexodon*, *N. tambuloides*, *N. peronii*, *N. thosaporni*, *N. nematophorus*, *N. bipunctatus*, *N. marginatus*, and *N. furcosus*). In addition, we included fifteen specimens belonged to four species (*N. hexodon*, *N. japonicus*, *N. furcosus*, *N. tambuloides*) from Vietnam and seven specimens of one species (*N. japonicus*) from Pakistan as regional conspecific comparison with our

samples and a specimen identified as *Scolopsis vosmeri* as an out-group. All samples were photographed and have been kept as voucher specimens at Zoological Museum in Biodiversity Centre, Universiti Sains Malaysia after preservation in 95% alcohol.

COI divergence analysis

A total of 153 sequences (127 sequences from current study and 26 sequences retrieved from NCBI and BOLD) of various species from genus *Nemipterus* were analyzed in this study. All specimens were successfully amplified and cross referenced to GenBank and BOLD systems. Most sequences showed > 98% identity to the species sequences from both databases as had been morphologically determined. However, three samples which had been morphologically been classified as *N. japonicus* were genetically identified as *N. nemurus* making up a total of 10 species compared to only 9 species from the initial morphological identification. Overall, a consensus length of 639 base pairs was used for analysis. The investigated sequences clustered into several haplotypes for each putative species generating a combined 52 unique haplotypes (Table 3). We found 294 variable positions with 255 parsimonious sites (39.9%) for further use in constructing phylogenetic tree. No insertions/deletions, stop codon or heterozygous sites were detected. Hence, all of the amplified sequences represent functional mitochondrial COI sequences.

Table 3. Number of samples, haplotypes and haplotype ID of *Nemipterus* spp. analysed in the study

	N. hexodon	*N. japonicus*	*N. tambuloides*	*N. bipunctatus*	*N. furcosus*	*N. nematophorus*	*N. marginatus*	*N. thosaporni*	*N. peronii*	*N. nemurus*
No. of samples	9	47	4	12	25	2	10	11	4	3
No. of haplotypes	4	22	2	4	5	2	3	5	2	3
Haplotype ID	H21, H22, H23 H24,	H28,H29, H30,H31 H32,H33, H34,H35 H36,H37, H38,H39 H40,H41, H42,H43 H44,H45, H46,H47 H48,H52	H1 H2	H3, H4 H5, H6	H16, H17 H18, H19, H20	H7,H8	H25,H26, H27	H11, H12 H13, H14 H15	H9, H10,	H49,H50, H51

Table 4. Inter-specific and intra-specific genetic distances of *Nemipterus* spp.

Species name	*N. japonicas*	*N. nemurus*	*N. furcosus*	*N. tambuloides*	*N. marginatus*	*N. thosaporni*	*N. hexodon*	*N. bipunctatus*	*N. nematophorus*	*N. peronii*	*N. zysron*	*N. mesoprion*	*N. virgatus*	*N. bathybius*	*S. vosmeri* (outgroup)
N. japonicus	**0.027***														
N. nemurus	0.175	**0.034***													
N. furcosus	0.167	0.183	**0.005**												
N. tambuloides	0.169	0.218	0.171	**0.001**											
N. marginatus	0.136	0.212	0.184	0.209	**0.015**										
N. thosaporni	0.188	0.192	0.187	0.155	0.193	**0.018**									
N. hexodon	0.195	0.208	0.169	0.143	0.213	0.137	**0.018**								
N. bipunctatus	0.200	0.216	0.162	0.138	0.205	0.149	0.131	**0.002**							
N. nematophorus	0.179	0.218	0.170	0.141	0.206	0.120	0.144	0.142	**0.023***						
N. peronii	0.164	0.161	0.100	0.184	0.187	0.206	0.216	0.178	0.199	**0.024***					
N. zysron	0.186	0.211	0.171	0.148	0.198	0.116	0.120	0.150	0.110	0.187	**0.000**				
N. mesoprion	0.183	0.199	0.173	0.145	0.195	0.105	0.108	0.144	0.110	0.183	0.013**	**0.003**			
N. virgatus	0.178	0.200	0.150	0.126	0.197	0.138	0.131	0.131	0.111	0.196	0.122	0.121	**0.000**		
N. bathybius	0.172	0.225	0.183	0.133	0.192	0.112	0.117	0.144	0.105	0.198	0.082	0.076	0.105	**0.000**	
S. vosmeri (outgroup)	0.244	0.230	0.237	0.241	0.245	0.245	0.236	0.236	0.265	0.232	0.245	0.233	0.262	0.248	**0.000**

Note: Value in bold represents intra-specific genetic distance values, * indicates high intra-specific distance value, ** minimum inter-specific genetic distance obtained

Genetic distances

Table 4 summarizes the genetic distances within and between genera and species. Intra-specific distances ranged from 0.0-3.4% while inter-specific distances ranged between 1.3-21.8%. Intra-specific distances in *N. japonicus*, *N. nemurus*, *N. nematophorus* and *N. peronii* were > 2% (2.7%, 3.4%, 2.3% and 2.4% respectively). Low interspecific genetic distance of 1.3% was obtained between *N. mesoprion* and *N. zysron* obtained from GenBank (EF609557, EF609581, JX866609, and JN992287).

Phylogenetic analysis

We used the 52 newly generated unique haplotypes and 26 additional sequences from GenBank and BOLD which comprised of 14 species of threadfin breams to construct a Neighbor-Joining tree. All samples generated from this study were clustered into their presumed species (Fig 2) according to BOLD data system and generally GenBank, including *N. nemurus*, *N. nematophorus* and *N. peronii* which have very small sizes. All haplotypes clustered with their respective species. However, a few sequences from GenBank require further taxonomic clarification. For instance, the presumed *N. japonicus* specimen, JN992288 had a low genetic distance with *N. nemurus* was more closely related to the basal taxon, *N. nemurus*. Furthermore, GenBank sequences, *N. zysron* and *N. mesoprion* with a genetic distance of only 1.3% clustered together.

We found two major clusters clades dividing *N. japonicus*. Cluster 1 Clade 1 was comprised of samples from the Arabian Sea (Indian Ocean) and Straits of Malacca. Cluster 2 Clade 2 clustered all South China Sea, Sulu Sea and Celebes Sea individuals. The intra-specific distance between the two clusters clades was 2.7%. Despite the smaller sample sizes, a similar trend was observed in *N. nematophorus* and *N. bipunctatus*, *N. hexodon*, *N. furcosus*, *N. marginatus* where samples from the Indian Ocean and the three seas (South China Sea, Sulu Sea and Celebes Sea) grouped into two distinct clusters, both of which are interspersed with specimens from the Straits of Malacca. For other species, samples were clustered in mixed localities. However, sample sizes were low compared to *N. japonicus*.

Discussion

Initially we identified 127 samples of *Nemipterus* into nine species through morphological key (Russell 1990). However, using DNA barcoding approach, three putative *N. japonicus* were re-classified and identified as *N. nemurus* (KTK6, KCH5 and KD05). Such misidentification is not an uncommon occurrence for morphologically similar species. Often diagnostic characters of specimens are lost due to preservation method or handling during capture. We did not observe the golden yellow stripes which extend from posterior nostril through eye and from upper lip to lower eye, characteristic of *N. nemurus*. Instead, a pale golden-yellow stripe along the body from behind head to base of caudal fin which is typical of *N. japonicus* was observed during the morphological inspection. We suspect found that

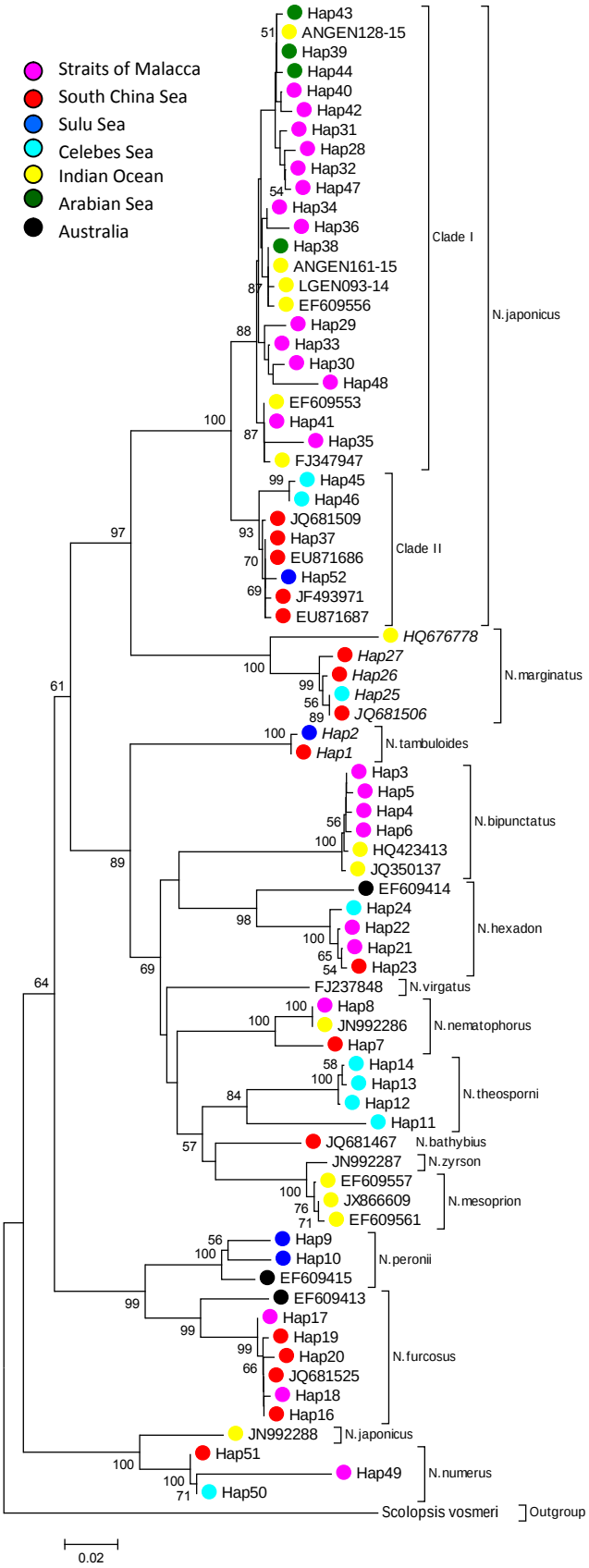

Figure 2. Neighbor-joining tree of species of *Nemipterus* using the cytochrome oxidase c subunit I

this coloration is unstable as specimens may have been out of the waters for a longer period. Alternatively, hybridization between the two species could have occurred, reflecting the maternal origin of the three specimens. This has proven the utility of the DNA barcoding method in identifying *Nemipterus* species when diagnostic morphological characters are not perfect or complete. Similarly, Becker et al. (2011) also reported that this approach can be used for fish identification for various stages of the life cycle and forms of seafood product whole fish, fillets, fins, fragments, juveniles, larvae, eggs, or any properly preserved tissue available. Such advantages can aid in fisheries management and conservation (Moura et al. 2008) as well as to prevent seafood fraud.

Several sequences from GenBank require further taxonomic validation. This include a voucher specimen of *N. japonicus* (ID JP992288) that clustered with *N. nemurus*, similar to our observation for the three samples and we believe is attributable to the same factors discussed. Becker et al. (2011) identified errors in FISH-BOL barcode data. He predicted that contradiction in identifications of same taxa can be seen when many laboratories are working on similar taxa and this misidentification of voucher specimens can also have serious implications for end users of reference libraries. Likewise, the genetic distance value calculated between *N. mesoprion* and *N. zysron* which atypically low (1.3%) between marine species and might also be due to misidentification.

Higher than typical marine fish intraspecific divergence of 2.7% and 3.4% were observed in *N. japonicus* and *N. nemurus*. Environmental heterogeneity and life-history traits could be factors elevating the variability. A similar divergence value of 2.7% had been previously reported (Ning et al. 2015) in *N. japonicus* populations from the Indian Ocean and West Pacific Ocean which was attributed to presence of cryptic species. Lim et al. (2014) reported that while the populations of *N. japonicus* in the Straits of Malacca were panmictic from the Perlis waters in the Northwest to Kuala Sedili in the Southeast, a distinct cluster clade was observed in the South China Sea population of Tok Bali. In the current study clustering of *N. japonicus* in the NJ tree into two separate clusters clades separating the South China Sea, Sulu Sea and Celebes Sea from Indian Ocean (populations from Pakistan) suggests limited sharing gene pool between the two regions. Haplotypes retrieved from GenBank belonging to South China Sea also clustered in same Cluster II Clade II. Interestingly, haplotype sharing was observed in the Straits of Malacca with only Indian Ocean populations. Whereas the South China Sea populations are isolated.

The parallel findings with Ning et al. (2015) and Lim et al. (2014) signifies the presence of a genetic barrier between the Indian Ocean and South China Sea for this demersal fish with the Straits of Malacca being the focal point of the two groups. Although on a smaller sizes, a detailed inspection showed that the same trend was also observed in *N. japonicus, N. bipunctatus, N. hexodon, N. furcosus, and N. marginatus.* This is in contrast to the lack of structuring of the pelagic Indian mackerel, *Rastrelliger kanagurta* (Akib et al. 2015). Ravitchandirane et al. (2012) reported genetic divergence values sufficient to differentiate various species of threadfin breams. Future studies on increased number of species and populations within this region are required to verify this.

To conclude, this study has contributed important data for the management of the threadfin breams in the Malaysian waters in the aspects of precise identification and genetic variability. Furthermore, it has provided additional data to the major databases of GenBank and BOLD. We confirm that DNA barcoding can efficiently diagnose genetic differences and genetic distances among and within species as well as resolving the issue of ambiguousness in catch identification. We found BOLD as an authentic genetic sequence library of voucher specimens. We recommend further validation of GenBank sequences with respect to their voucher specimen to prevent future misidentification of fish species.

ACKNOWLEDGEMENTS

This investigation was supported by Grant No 1002/BIOLOGI/910317). We are very grateful to Khawar Pervez Awan (Fisheries Department, Sindh, Pakistan), Zahoor Abbasi and Anees Soomro (Karachi Fisheries Harbour Authorities) for providing us samples from the Indian Ocean. We would also say like to thank the Department of Fisheries Malaysia for help identification of fish specimens. Finally, we thank members of Lab 308 School of Biological Sciences, Universiti Sains Malaysia for their aid on various aspects of this study.

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