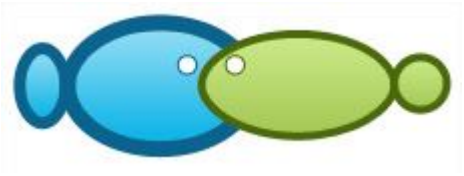

DNA barcoding of nomei fish (Synodontidae: *Harpadon* sp.) in Tarakan Island, Indonesia

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Abstract. Nomei or "lembe-lembe", a local name for fishes of the family Synodontidae, is an economically important fish in the city. The lack of detailed and precise reports of this fish has caused extinction status of nomei species in its distribution range in Tarakan. These were sequenced for the 618-bp region of the mitochondrial cytochrome c oxidase sub unit I gene (COI). The aim of this research was to evaluate the genetic variation and phylogenetic comparison among nomei fish in Tarakan Island, Indonesia. The results of alignment showed 12 substitutions of nucleotides bases, which consisted of 12 transitions with no transversions, insertions and deletions. The phylogenetic tree resulting Neighbour Joining (NJ), Maximum Parsimony (MP) analysis and network analysis showed that Nomei fish from Amal and Juata samples were clustered in one group. Divergent sequence of nomei fish from Amal and Juata (average 0.354±0.008) in comparison of *Harpadon nehereus*. This novel of Nomei barcode will help provide a database for Indonesian fish, to be recorded using the Barcoding of Life Data System.
Key Words: nomei fish, phylogeny, genetic diversity, COI gene, Tarakan.

Introduction. Synodontidae is a family of Nomei fish of the Order Aulopiformes and Class Actinopterygii. The fish is generally distributed from West coast of Sumatra (Aceh, Lampung, Bengkulu, Padang, Pariaman), Eastern part of Sumatra (South Sumatra), Java (West Java), West Kalimantan (Nugroho et al 2014), East Kalimantan (Tarakan), South and Southeast Sulawesi, Maluku and Irian Jaya, Arafuru Sea, as well as along the South China Sea coast. In the Tarakan, *Harpadon* sp. is locally called as "nomei" or "lembe-lembe" (Nugroho & Rahayu 2015).

The current status of the fish is quite alarming as local Fisherman and Regional Fisheries in Tarakan revealed that the average catch over the years has greatly decreased. Limited studies have been conducted on this species to date. Some of these studies involved studying of growth analysis and age structure (Firdaus et al 2013), bio ecology and reproduction (Saleh 2007). In this regards, some limited genetic studies have also been carried out, including identification and phylogenetic study of *Harpadon* sp. based on *16S rRNA* gene (Nugroho & Rahayu 2015). Nowadays, one of the best choices to protect a species is to preserve its genetic resources and genetic diversity besides the preservation of the ecosystem (Hedrick 2001).

Presently, the efforts are underway to conserve and protect the remaining population because of this rearing techniques and culture of this fish living in the sea are difficult to develop. Considering the lack of any information of genetic analysis of this fish, in the present research we have tried to confirm the presence of nomei fish species in Tarakan using the analysis of partial sequence of mitochondrial COI gene. We would thus be able to identify the genetic analysis and genetic diversity of this species. Besides that, the identification of a species is needed as the source of information on species genetic richness and the relationship between inter-states species from the family Synodontidae.

Species identification through genetic analysis is almost always efficiently solved by the use of a standardized molecular approach such as DNA barcoding using COI gene

(Hebert et al 2003; Hajibabei et al 2007). The most commonly used gene that uses as the barcoding marker is protein coding gene cytochrome-c Oxidase I with base length 648 bp (Zhang & Hewitt 1997). The gene of COI provides very fast and accurate method for marker to identify various taxa and reveals several animal groups that its level of taxonomy has not been acknowledged yet. In Indonesia, Nomei fish has not been described and there is no database found, thus it is necessary to perform such study.

Material and Method

Description of the study sites. Location of sampling for *Harpadon* sp. was in two sites, i.e., at Amal Sea, Tarakan, Indonesia at coordinate 03°24.889" S and 117°42.352' E, and Juata Sea at 03°31.666"S and 03°31.666' E (Figure 1). The sampling was conducted on July 2014. Tissue extraction involved cutting a small piece of muscle tissue from the dorsal anterior side of the fish and preserved in 70% of alcohol. A total 4 samples were gathered from the sampling sites (Figure 1): two nomei fish were collected from Amal and two from Juata Sea. *Harpadon nehereus, Harpadon microchir, Harpadon squamosus,* and *Saurida elongate* were used as a sister group, while *Saurida gracilis* and *Synodus variegatus* marker sequences from GeneBank were used as outgroup sequences for the genetic analysis. DNA barcode of this sample was analyzed in the molecular biology laboratory, Department Biology, State of University of Malang, Indonesia.

Figure 1. Geographic location of collected samples.

DNA extraction. Total genomic DNA from each sample was extracted using *DNA Isolation Mini Kit (ROCHE)* with some modification (Nugroho et al 2015; Nugroho & Rahayu 2017), and this was used as the template to amplify a 615 bp fragment of the COI gene using the primers *fishF2_tl*: 5'-TCGACTAATCATAAAGATATCGGCAC-3' and *FishR2_tl*: 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3' (Zhang 2011). Quantitative test on total DNA was conducted by using UV spectrophotometer NANO DROP 2000.

PCR cycle, and sequencing. PCR reaction was carried out in a thermal cycler as follows: 1 min of initial denaturation at 94°C; 40 cycles of 45 s of denaturation at 94°C, annealing at 45°C for 45 s, and extension at 72°C for 1 min 30 s; and final extension at 72°C for 10 min. PCR composition for COI gene with total solution 50 µL (according to the procedure of iNtRON Biotechnology), i.e., 2x PCR Master Mix Solution 25 µL, DNA template 1-2 µL, Primer F (10 pmol μL^{-1}) 1 µL, Primer R (10 pmol μL^{-1}) 1 µL, and double-distillated water (ddH_2O) 21-22 µL. The PCR products were viewed on 1% agarosa gels stained with ethidium bromide. The purified DNA products were sent to Macrogen Inc. in Seoul, Korea for bidirectional sequencing. The DNA sequence analyser used was 373XL

DNA analyzer with BigDye v3.1. Each DNA specimen was sequenced for the 615-bp of the COI barcode region

Data analysis. DNA sequencing was performed at First Base, Malaysia. The phase of genetic analysis was the chromatogram checking with sequencer software to be analyzed further by using DNA STAR to display the chromatogram sequence and create consencus (combine the forward and reverse primer). After that, the Basic Local Alignment Search Tool (Altschul et al 1997) in the NCBI database was used to determine the similarity of sequences sample. Before alignment, every sample has to be translated into protein (without any stop codon in the middle) by using SeqMan DNASTAR. COI sequence of other Synodontidae species available in GenBank and Barcoding of Life Data Systems (BOLD) (Ratnasingham & Hebert 2007) were included in subsequent analysis. These included COI sequences of *Harpadon nehereus, Harpadon microchir, Harpadon squamosus, Saurida elongata, Saurida gracilis,* and then *Synodus variegatus* as outgroup species from genebank (Table 1). The construction of phylogenic topology used the neighbor joining and maximum parsimony methods with 1000 bootstrap repetitions using the Kimura 2-parameter (K2P) distance model. The COI sequences were submitted to the gene bank and BOLD System.

Table 1

Variation of nucleotide base of *Harpadon* sp. with comparative species

Taxon	*Nucleotide substitution*											
	13	*42*	*60*	*87*	*150*	*159*	*162*	*219*	*244*	*255*	*354*	*549*
Harpadon nehereus 8	A	C	A	T	A	A	C	C	C	A	T	T
Harpadon nehereus 3	●	●	●	●	●	●	●	●	●	●	●	●
Harpadon sp. Juata 2	G	T	G	C	G	G	●	T	T	G	●	C
Harpadon sp. Juata 1	G	T	G	C	G	G	●	T	T	G	●	C
Harpadon sp. Amal 2	G	T	G	C	G	G	T	T	T	●	●	C
Harpadon sp. Amal 1	G	T	G	C	G	G	T	T	T	●	●	C
Harpadon nehereus 4	G	T	G	C	G	G	●	T	T	●	C	C
Harpadon nehereus 1	G	T	G	C	G	G	●	T	T	●	C	C
Harpadon nehereus 6	G	T	G	C	G	G	●	T	T	●	C	C
Harpadon nehereus 2	G	T	G	C	G	G	●	T	T	●	C	C
Harpadon squamosus 2	C	●	●	C	●	G	●	●	●	●	C	C
Harpadon squamosus 3	C	●	●	C	●	G	●	●	●	●	C	C
Harpadon squamosus 1	C	●	●	C	●	G	●	●	●	●	C	C
Harpadon microchir	C	●	●	C	●	G	●	●	●	●	C	C

Note: ● it means conserved sequence.

Results and Discussion. The alignment results showed that the COI sequence of nomei fish from Amal and Juata with its related species (*H. nehereus*, *H. microchir*, and *H. squamosus*) was 618 bp. Using BLASTn, DNA sequences of the samples using COI gene are approximately 97% identical with the *H. nehereus* isolate F00045. The composition of nucleotides of *Harpadon* sp. from Amal and Juata Seas with comparison species are A = 20.39%, T = 30.91%, G = 20.55% and C = 28.15%. Based on this composition of nucleotides it is showed that the GC content is less than AT nucleotides. Protein translation resulted from 618 bp is 206 amino acids. The translation results indicate there is no pseudogene on the sequence of amino acids, thus the sequence of COI gene is very strongly used as a standard barcode for the identification of nomei fish in Tarakan waters, North Kalimantan.

The result of alignment on 18 sequences COI gene of nomei fish from Amal and Juata with references species, and out group showed 12 substitutions of nucleotide bases (Table 2). The substitution of these nucleotide bases consisted of 12 transitions, no tranversion and no insertion and deletion. The authomorphism of nucleotide base was used as a marker to distinguish species among the *Harpadon* genus. This character was showed as much as 44 nucleotide bases (Table 3). The automorphism of nucleotides belonging to *H. nehereus* are found in number of 24, the sample (*Harpodon* sp.) with

sister species (*H. nehereus*) has a nucleotide base G (Guanin), while *H. squamosus* and *H. microchir* have nucleotide A (Adenin) bases (Table 3). The automorphism character is a unique character possessed by only one species, which can be used to differentiate with other species. The automorphism character may also strengthen the position of the sample taxon as the species *H. nehereus*.

Construction of phylogenetic topology on the sequence COI gene sample was constructed based on the method of Neighbour Joining (NJ) (Figure 2) and Maximum Parsimony (MP) (Figure 3) with calculation of Kimura 2 parameters. The phylogenetic tree between samples with reference species showed two large clusters of the genus *Harpadon* supported by bootstrap value of NJ 100 / MP 99. Nomei fish (*Harpadon* sp.) Amal 1 and Amal 2, and Juata 1 and Juata 2 are one cluster with *H. nehereus* (4, 1, 6, and 2) from Gene Bank supported with bootstraps NJ 100% and MP 84%, and still belong to sister group with *H. squamosus* and *H. microchir.* The calculation method employed supports the consistency that nomei fish from Amal and Juata form one species with *H. nehereus*, related species with *Harpadon microchir* and *Harpadon squamosus*, sister species with *Saurida elongata* and *Saurida gracilis*, but still in one cluster group and separated group with *Synodus variegatus* 1 and *Synodus variegatus* 2.

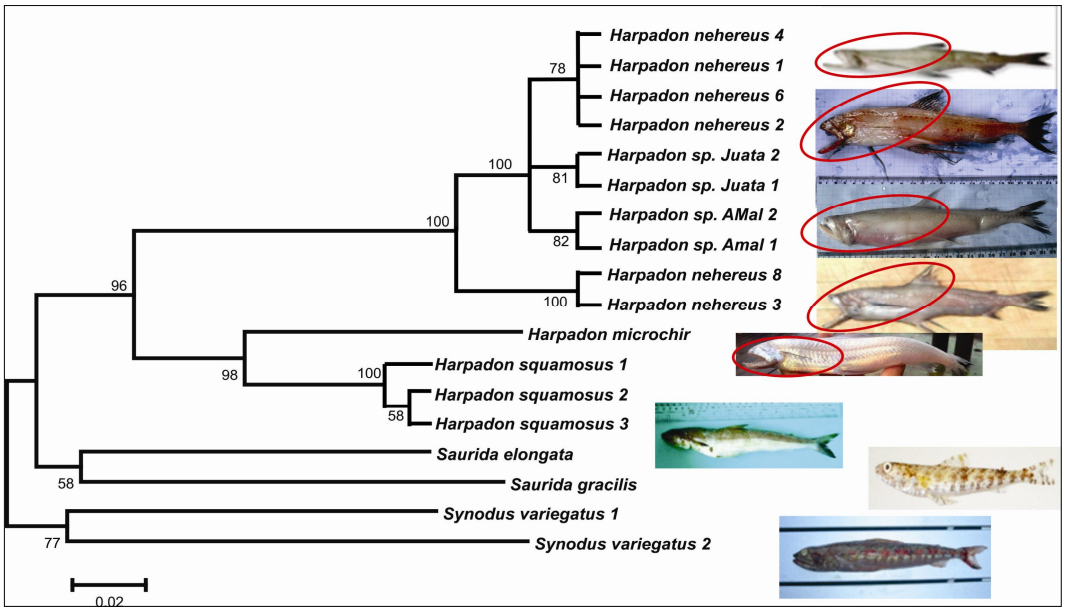

Figure 2. Phylogenetic topology used Neighbour Joining method with 1000 bootstrap repetitions.

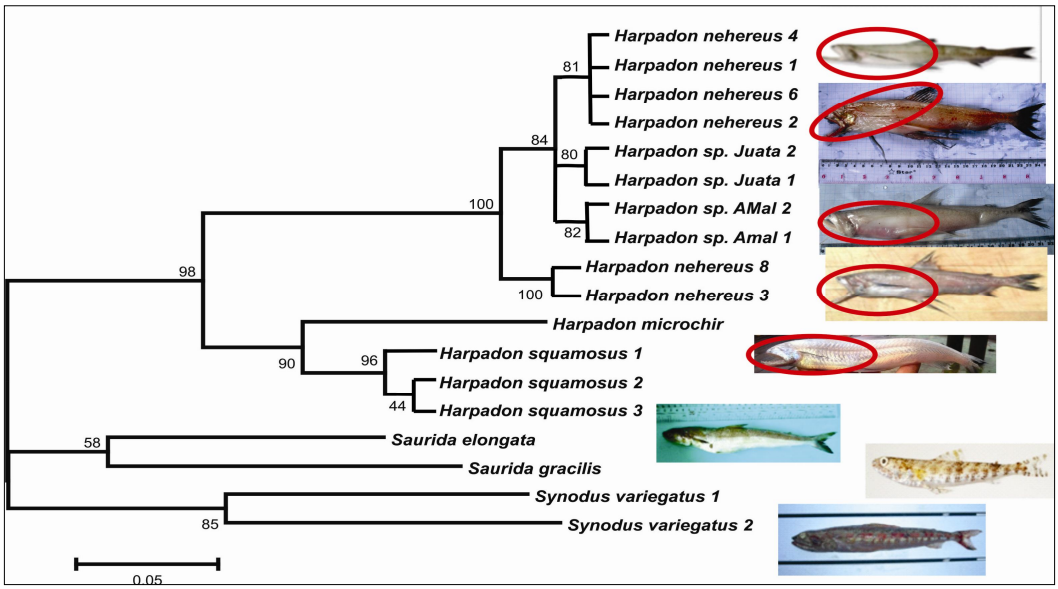

Figure 3. Phylogenetic topology used Maximum Parsimony method with 1000 bootstrap repetitions.

Table 2

Automorphism of nomei fish by COI gene (*Harpadon* sp.) with sister species (*Harpadon nehereus*)

Taxon	Character of base automorphism																																										
	24	28	45	89	90	102	165	177	198	216	249	261	267	276	279	285	297	312	328	336	339	348	354	360	363	369	378	381	412	444	456	483	501	502	503	532	537	555	558	565	578	594	612	618
Harpadon nehereus 8	G	A	G	T	T	C	G	T	C	G	C	T	T	G	A	G	T	C	C	G	A	T	T	C	T	T	C	C	T	C	T	C	A	C	G	T	C	C	A	T	T	C	C	T
Harpadon nehereus 3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon sp. Juata 2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon sp. Juata 1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon sp. Amal 2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon sp. Amal 1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon nehereus 4	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon nehereus 1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon nehereus 6	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon nehereus 2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Harpadon squamosus 2	A	C	A	A	C	T	A	C	T	A	T	C	C	A	C	A	C	T	G	A	G	A	C	A	C	C	T	T	C	T	C	T	T	T	A	C	T	T	T	C	C	T	A	C
Harpadon squamosus 3	A	C	A	A	C	T	A	C	T	A	T	C	C	A	C	A	C	T	G	A	G	A	C	A	C	C	T	T	C	T	C	T	T	T	A	C	T	T	T	C	C	T	A	C
Harpadon squamosus 1	A	C	A	A	C	T	A	C	T	A	T	C	C	A	C	A	C	T	G	A	G	A	C	A	C	C	T	T	C	T	C	T	T	T	A	C	T	T	T	C	C	T	A	C
Harpadon microchir	A	C	A	A	C	T	A	C	T	A	T	C	C	A	C	A	C	T	G	A	G	A	C	A	C	C	T	T	C	T	C	T	T	T	A	C	T	T	T	C	C	T	A	C

Note: • it means conserved sequence.

Table 3

Estimates of evolutionary divergence between species using K2P distance species

	Harpadon nehereus	*Harpadon* sp. Amal	*Harpadon* sp. Juata	*Harpadon squamosus*	*Harpadon microchir*
H. nehereus	0.455±0.02				
Harpadon sp. Amal	0.354±0.008				
Harpadon sp. Juata	0.354±0.008	0.3±0.002			
H. squamosus	17.3±0.018	17.8±0.023	17.46±0.021		
H. microchir	21.3±0.019	21.8±0.022	21.5±0.025	11.6±0.003	0.23±0.002

The genetic relationship can be shown by the phylogenetic approach. The established cladogram has proved that *Harpadon* sp. Juata and Amal are the same species (monophyletic) with *H. nehereus* (1, 2, 4 and 6). *H. nehereus* 8 and *H. nehereus* 3 are in the different clade and separated cluster with *Harpadon* sp. Juata and Amal. The phylogenetic topology generated by MP or NJ (Figures 2 and 3) method shows an identical topology with little difference only to the value of bootstraps. The genetic relationship of *Harpadon* sp. Amal and Juata Sea above was supported by the result of genetic relationship of *Harpadon* sp. with 16S rRNA gene which showed that this fish in one sister group (monophyletic) (Nugroho & Rahayu 2015).

The divergent sequence between nomei fish (*Harpadon* sp.) Amal with *H. nehereus* (1, 2, 4 and 6) is 0.346-0.362 (average 0.354±0.008); nomei fish (*Harpadon* sp.) Juata with *H. nehereus* (1, 2, 4 and 6) (average 0.354±0.008). The divergent sequence between nomei fish (*Harpadon* sp.) Amal with *H. squamosus* is 17.77-17.82 (average 17.8±0.023), whereas the divergent sequence between nomei fish (*Harpadon* sp.) Amal with *H. microchir* is 21.78-21.82 (average 21.8±0.022). From the calculation results it is showed that the genetic distance of *Harpadon* sp. Amal and Juata from Tarakan, Indonesia has high similarity sequence. It means that the sample was intra-species to *H. nehereus*.

The mean K2P distance within species was 0.458% while that between *Harpadon* species was 17.23%. The mean intraspecific distance is much lower than the 3% thereshold suggested by Hebert et al (2003). The mean interspecies distance is as high as the interspecific distance observed by Ward et al (2005). In this study, nomei fish from Amal and Juata, Indonesia showed very low sequence divergence. One was between *H. nehereus* from Amal and *H. nehereus* from database. It means that the sample was intraspecies with *H. nehereus* (1, 2, 4 and 6) group.

The value of genetic distance between sample (Nomei fish from Amal and Juata waters) less than 3%, was indicated that nomei fish (*Harpadon* sp.) Amal and Juata are intraspecies with *H. nehereus* from the genebank. This is supported by the research of Freitas et al (2011), that the genetic distance of *Salminus* fish in the Brazilian river is calculated on the basis of K2P, the value ranging from 0.8 to 3.5% in species, and 4.6-7.1% in the genus. The genetic distance in fish ranges between 3% and 6%, the range was able to distinguish species belonging to one genus. *Salminus* species that have a 3.5% range in species include cryptic species.

The median joining network analysis of 10 individuals of the genus *Harpadon* was performed using the Network 4.1.0.8. Median Joining network makes a description of the variation of nomei fish (*Harpadon* sp.) Amal, Juata, with reference species to 9 haplotypes and divided into 3 haplogroups. Haplotype 1, 2 and 3 are the *H. squamosus* group; Haplotype 5 is *H. microchir*; and the *H. nehereus* group is divided into 5 haplotypes, including the nomei fish from Amal and Juata (Figure 4A). The haplotype network showed that Nomei fish from Amal and Juata are one group with *H. nehereus*. The haplotype network using the Median Joining network method shows that nomei fish (*Harpadon* sp.) from Amal and Juata was one group with *H. nehereus* from South China (Zhang 2011). Each fish has a different haplotype, not homologous. *Harpadon* sp. Amal 1 and 2 make their own haplotype of haplotype 7, while *Harpadon* sp. Juata 1 and 2 also formed their own haplotype in haplotype 9. Haplotype of nomei fish (Amal and Juata) has potential as a new haplotype, because it is not homologous with reference species from

the gene bank, but still in one cluster. This grouping is based on substitution differences caused by years of geographic isolation. Nucleotide base substitution between *Harpadon* sp. Amal 1 and 2 with *H. nehereus* differ in base number 162, namely *H. nehereus* is indicated by a cytosine base, whereas *Harpadon* sp. Amal 1 and 2 have a thymine base. The same is true for *Harpadon* sp. Juata 1 and Juata 2 which have a cytosine nucleotide base, while *H. nehereus* on base 225 has a guanine base.

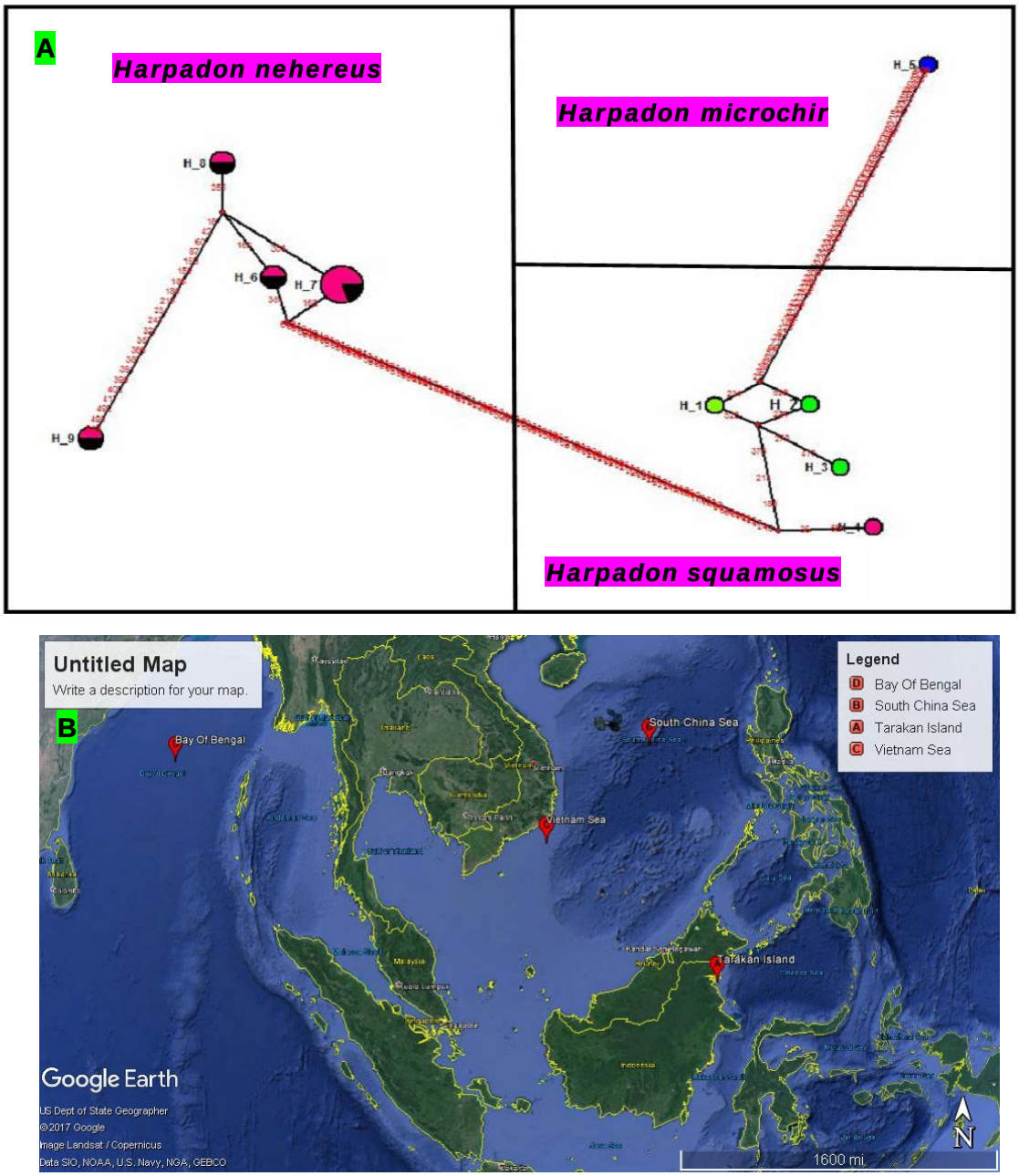

Figure 4. A. Haplotype network of 9 haplotypes based on COI gene sequence. B. Geographical distribution of haplotype fish of the genus Harpadon. The haplotype is represented by different circles and patterns, the number of individuals is represented by different circular forms (large = 3 individuals, medium = 2 individuals, and small = 1 individual). The branching between haplotype is indicated by substitution based on the alignment position of the COI gene sequence (Base Map 2016 & Landsat OLI 2016).

The related relationship of nomei fish from Amal and Juata with *H. nehereus* from South China and Vietnam from genebank can be attributed to the abundance of demersal fish, such as subfamily Harpadontinae in tropical continental exposures that resemble the Indo-Pacific (Figure 4B). This is supported by the opinion of Longhurst & Pauly (1987)

which state that at the beginning of the tertiary era, the West Mediterranean Atlantic fauna was part of the Tethys Sea which is very similar to the fauna of the West Indo-Pacific. The historical factor of the likeness of the Tethys Sea fauna and Indo-Pacific Sea allowed the nomei fish from the waters of Amal and Juata to be a species and closely related to the *H. nehereus* found in the sea of southern China and the sea of Vietnam. The results of this study also show that there are some variations of *H. nehereus* species, due to environmental factors and the isolation in different places that have lasted thousands of years.

Our results demonstrate that the COI gene is an efficient marker to distinguish species in different populations of genus *Harpadon* in Indonesia. Based on this research result, COI gene can be used as a basic and applied markers to identify species and genetic relationship of genus *Harpadon* from Tarakan, Kalimantan. This study was expected to be published in Gene Bank and BOLD System, thus it can be used for the efforts of conservation and management of nomei fish in Indonesia waters. It also provides new information on the molecular-based taxonomy data.

Conclusions. In this study, nomei fish from Amal and Juata, Indonesia showed very low sequence divergence. Divergent sequence of nomei fish from Amal and Juata (average 0.354±0.008) in comparison with *H. nehereus*. The phylogenetic tree resulting after NJ and MP analysis and network analysis showed that *Harpadon* sp. from Amal and Juata samples were clustered in one group with *Harpadon nehereus* from the genebank. The value of genetic distance between sample (nomei fish from Amal and Juata waters) less than 3%, indicated that nomei fish (*Harpadon* sp.) Amal and Juata are intraspecies with *H. nehereus* from the genebank.

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