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by Dwi Anggorowati Rahayu Dkk

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Research Article

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Molecular characterization of genus Tor from Indonesia based on 16S rRNA

Dwi Anggorowati Rahayu[1*], Endik Deni Nugroho[2], Rodiyati Azrianingsih[3], Nia Kurniawan[3]

[1*]Department of Biology, Universitas Negeri Surabaya, Surabaya, Indonesia
[2]Biology Education, Nahdlatul Ulama Pasuruan Institute of Technology and Science, Pasuruan, Indonesia
[3]Department of Biology, Brawijaya University, Malang, Indonesia
Email: dwirahayu@unesa.ac.id*, endik@itsnupasuruan.ac.id, rodiyat@ub.ac.id, wawan@ub.ac.id

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ABSTRACT

Genus Tor is Indonesia's endangered local fish however, the reports on this species from South East Asia is currently limited. This study conducted to characterize molecular genetics and phylogenetic relationship of Tor fish from East Java, West Kalimantan, Padang and North Sumatra from BPBAT Bogor collection using a partial sequence of mtDNA 16S rRNA. A total of ten samples of Genus Tor were collected, then identified based on morphological characters, and the identification was confirmed using molecular data. PCR amplicons at 542 bp in length. The construction of phylogenetic topology was made based on ML and NJ method using Kimura-2 parameter model. Based on the phylogenetic topology showed that Tor fish from Pasuruan are closely related to Tor duoronensis from Padang with a bootstrap value of 66%, while Tor duoronensis fish from North Sumatra and Tor tambra from West Kalimantan are in separated clusters. This finding also contributes to the differentiate Genus Tor from Indonesia based on polymorphic sites.

Keywords: 16S rRNA; genus Tor; mitochondrial DNA; phylogenetic

How to Cite

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INTRODUCTION

Genus Tor is known as local Indonesian fish. Based on the Red List of Endangered Species published by IUCN in 1990 there were 29 species of fish from Indonesia, including all the Genus Tor (Kottelat & Whitten, 1994). Haryono & Tjakrawidjaja (2006) stated that in Indonesia there were four species of genus Tor fish namely *Tor tambroides* Blkr, *Tor douronensis* (C.V.), *Tor tambra* (C.V.) and *Tor soro* (C.V.). Weber & Beaufor (1916) previously named this fish Labeobarbus, and distinguished the type based on lobe size on the lower lip. Previous studies have reported by Kottelat & Whitten in 1994, the types of fish from the Genus Tor were unclear taxonomically and systematically.

In her case study of identification morphological of genus Tor, Rahayu and Nugroho (2014) and Rahayu et al. (2016) identifies diagnostic characters of this fish. There were: fleshy lips, lower lip with or without a median lobe and uninterrupted post-labial groove; and dorsal fin with a scale. Other recognize Tor by the following characters: lower lip developed into a fleshy lobe or at least with two notches delimiting the usual position of the lobe; uninterrupted post labial groove While, the main character distinguishing the species of Tor Genus is the presence and size of lobus on the lower lip. In addition, the distinguishing character is complemented by comparison between the length of the third dorsal fin and the head of the length, the ratio between the length of the anal fin and dorsal. In 1916, Weber & Beaufort published taxonomic book of Fish in which they described that *Tor soro* haven't lobes on the lower lip; *Tor tambraides* have lobes until they reach the corner of the mouth; *Tor duoronensis* has short lobe and does not reach the corners of the mouth; while *Tor tambra* have lobes like *Tor duoronensis* but the length of the dorsal fin spines is shorter than the length of the head without a snout.

Genus Tor in Indonesia can be found in Banyu Biru Lake, Pasuruan Regency East Java Rahayu et al., 2016 and Rahayu & Nugroho (2014), North Sumatra, West Sumatra and West Kalimantan. Haryono and Tjakrawidjaja (2006) and Rahayu et al. (2016) identified that the population of Genus Tor in Indonesia is threatened with extinction due to deforestation and overfishing. Excessive exploitation activities will have an impact on the loss of local species. The waters of Java, Kalimantan and Sumatra are included in the Sundaland Region. These three regions are linked to the existence of ancient rivers around 17,000 to 20,000 years ago in the Pleistocene Era. The existence of this ancient river flow allowed this fish to swim towards the river flow connected to the ancient river to another location. This can be traced from the history of Java, Sumatra, and Kalimantan, which was formerly the area of the Great Sunda Exposure.

This paper will examine the phylogenetic of genus Tor were found in East Java, North Sumatra, West Sumatra and West Kalimantan based on 16S rRNA gene. Their genetic information at the molecular level will be helpful in the future study of genetic population. This effort is carried out to assist the conservation and cultivation of local species so that their existence will remain sustainable. Nowadays, one of the best choices to protect a species is to preserve its genetic resources and molecular characterization besides the preservation of the ecosystem (Nugroho & Rahayu, 2015). Molecular characterization are the basic concept for building classifications, understanding evolutionary relationships between organisms, and reaching correct identification methods (Eprilurahman et al., 2021; Glon et al., 2018; Skuza et al., 2016). The information of molecular characters can be used an effort to increase the number of populations and prevent inbreeding that is closely related because it will reduce the genetic diversity of species.

The first molecular phylogeny of Genus Tor was presented by Nguyen et al.(2008) using partial sequence of 16S rRNA to addresses question relation to genetic relationship among two Malaysian masheer species, *Tor tambraides* and *Tor duoronensis*. One alternative mapping of the genus Tor fish is by using the mtDNA 16S rRNA gene markers from the mitochondrial genome. DNA sequence of 16S rRNA showed high variability when compared to other DNA markers since is reported as highly conserved (Wong & Hanner, 2008). Dudu et al. (2011) published a paper in which they describe characteristic of this gene is very conserved, has a slow evolution rate, and has special universal primers to amplify specific regions as standard animal identification (Listyorini et al., 2011; Rahayu & Jannah, 2019). While, For the current study on Genus Tor mitochondrial 16S rRNA was used. These genes are conserved and non-coding in nature which played vital role in determination of new phylogenetic relationships and in checking reliability of earlier established taxonomic classification (Sharma et al., 2014). In the past to decade, mtDNA 16S rRNA gene has great potential used to explore the phylogenetic relationships of fishes at

varying taxonomic levels, e.g. At the order, family, subfamily, genus and species level (Nugroho & Rahayu, 2015; Rahayu et al., 2016). The results of this study are expected to complete the genetic information of Genus Tor with median lobe morphological characters that may support conservation in Indonesia and contribute to the understanding of the distribution of *Tor duoronensis* in East Java.

RESEARCH METHODS

This research is a descriptive explorative studied. A Sampling of Genus Tor are *Tor duoronensis* at Banyu Biru Lake, Pasuruan Regency, and reference species *Tor duoronensis* from Padang, *Tor tambraides* from West Kalimantan, and *Tor soro* from North Sumatra based on collection in BPBAT Cijeruk, Bogor, West Java. Samples were taken from pectoral fins and stored in 96% ethanol solution and labeled on each sample bottle. The research sample code is as follows (Table 1).

Table 1. Location and Sample Code of Genus Tor in This Study

Sample Code	Species	Location (native habitat)
TORD-1	*Tor duoronensis*	Padang
TORD-2	*Tor duoronensis*	Padang
TORS-1	*Tor soro*	North Sumatra
TORS-2	*Tor soro*	North Sumatra
TORT-1	*Tor tambraides*	West Kalimantan
TORT-2	*Tor tambraides*	West Kalimantan
SKRG-1	*Tor duoronensis*	Banyu Biru Lake, Pasuruan
SKRG-2	*Tor duoronensis*	Banyu Biru Lake, Pasuruan
TABR-1	*Tor duoronensis*	Banyu Biru Lake, Pasuruan
TABR-2	*Tor duoronensis*	Banyu Biru Lake, Pasuruan

Isolation DNA, the first step carried out in this study was the isolation of DNA using Kit Roche with several modifications. After obtaining total DNA, a quantitative test was immediately carried out using the NANO DROP 2000 UV spectrophotometer. The results of DNA isolation in each sample were taken as much as 3 µl and mixed with 1 µl of loading dye with 2 µl of sterile distilled water. The mixture of sample DNA, sterile distilled water and loading dye are included in the agarose gel well carefully using micropipette. Running DNA is done by connecting the cathode and anode at a voltage source of 100 V, 200 MA for 1 hour and this was used as the template to amplify a 542 bp fragment of the COI gene using the primers 16SarL 5'-CGCCTGTTTATCAAA AACAT-3' and 16SbrH 5'-CCG GTCTGAACTCAG ATCACGT-3' (Folmer et al., 1994). 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 a final extension at 72°C for 10 min. PCR composition of for 16S rRNA 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 µl, Primer R (10 pmol/ µl) 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 analyzer used was 373XL DNA analyzer with BigDye v3.1. Each DNA specimen was sequenced for the 542-bp of the 16S rRNA barcode region.

Genetic analysis, analysis of the 16S rRNA gene sequence is done using several computer programs, based on DNA Baser to make consensus sequences; BLAST to determine the compatibility of

the target gene with the Query obtained from Gene Bank, Clustal-X to make multiple alignments between the 16S rRNA gene samples and the *data base* of close relatives of the Cyprinidae family. The last step was the construction of phylogenetic topology using MEGA 5 computer program with Maximum Parsimony method using CNI (Close Neighbor Interchange) on random tree algorithm using Random addition sequence performed 10 repetitions, Minimum Evolution and Neighbor Joining with algorithmic calculation model Kimura-2 parameter, and Maximum Likelihood by using HKY (Hasegawa Kishino Yano) algorithmic calculation model. Tree evaluation is done using a bootstrap analysis of 1000 repetitions. Calculation of similarity values is:

Similarity percentage = (1 - Genetic Distance) x 100%

Composition modeling and differences in nucleotide bases of Tor species using the maximum likelihood method. Substitution of transitions and transversion of nucleotide bases is calculated by the Kimura 2 model. The polymorphism reported in this study was analyzed using MEGA 5. The substitution of nucleotide base transitions and transversion was calculated using the Kimura 2 model, while further analysis of Genus Tor with related species was analyzed for common haplotype sequences, using DNASP. V.5.0 computer program (Martins et al., 2013; Rozas, 1995). Furthermore, construction of the haplogroup for *Lingula* sp. with related species from GenBank were analyzed using median-joining method as well as the Network 4.1.0.8 computer program (Martins et al., 2013).

FINDING AND DISCUSSION

In this study, we have adopted the universal primer introduced by Folmer et al. (1994). The 16S rRNA gene has the potential to identify a genetic relationship in the middle category, namely family and genus level (Dudu et al., 2011; Pope et al., 2007; Rahayu et al. 2016). The 16S rRNA gene can be used to explain the genetic relationship of fish at different taxonomic levels, this is because these genes are highly conserved and have a slow evolutionary rate (Dudu et al., 2011). Figure 1 shows the results of 16S rRNA gene that was successfully amplified ± 542 bp.

Figure 1. Amplification results of gen *16S rRNA* Genus Tor with related species in agarosa gel 1% (DNA Marker 1 Kb). Information: 1) Sengkaring 1; 2) Sengkaring 2; 3) Tambra 1; 4) Tambra 2; 5) *Tor duoronensis* 1; 6) *Tor duoronensis* 2; 7) *Tor soro* 1; 8) *Tor soro* 2; 9) *Tor tambraides* 1; 10) *Tor tambraides* 2

Further alignment analysis was carried out to determine the homology level of the 16S rRNA gene sequence obtained between samples. Base composition analysis of mtDNA 16S rRNA showed that the GC content was found to be higher in the 16S rRNA gene. The polymorphic composition is A = 33.54%, C = 26.76%, G = 22.53% and T = 17.18%. Dominant bases are T and A bases. Alignment results showed

that 24 nucleotide base polymorphic site, such as 22 transitions, 2 transversions, and no indel (insertion and deletion). The Table 2 below illustrates nucleotide bases of transition in bases numbers of 17, 20, 30, 39, 56, 107, 129, 140, 149, 150, 151, 156, 159, 162, 183, 194, 318, 391, 394, 395 and 396, while nucleotide bases of transversion are shown in base number 389 and 392. One example of a nucleotide base undergoing transition can be shown in base number 17, *Tor duoronensis*-1 as Query having a nucleotide base A which is the same as other samples, except *Tor soro*-1 which has a base C (change from base A to C). Transversion can be found in number base 389, that is *Tor duoronensis*-1 has a nucleotide base T, whereas *Tor tambraides*-1 has a nucleotide base A (base change from base T to A).

Table 2. Polymorphic site of Genus Tor Based on 16S rRNA

CODE	17	20	30	39	56	107	129	140	149	150	151	153	156	159	162	183	194	318	389	391	392	394	395	396
TORD-1	A	C	T	T	C	G	G	G	A	T	T	C	C	C	T	A	A	A	T	A	G	G	T	T
TORD-2	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
TABR-1	•	-	•	•	T	•	A	A	•	•	•	T	•	•	•	•	•	G	•	•	C	•	•	•
TABR-2	•	T	•	•	T	•	A	A	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•
SKRG-1	•	•	•	•	T	•	A	A	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•
SKRG-2	•	•	•	C	T	-	A	A	•	•	•	A	T	•	•	•	•	•	•	•	•	•	•	•
TORS-1	C	-	•	C	•	A	A	A	G	C	C	•	•	•	•	•	G	•	•	•	•	•	•	•
TORS-2	•	T	C	•	•	A	A	A	G	C	C	•	•	T	G	•	G	•	•	•	•	•	•	•
TORT-1	•	T	C	•	•	A	A	A	G	C	C	T	•	•	•	G	•	•	•	•	•	T	C	G
TORT-2	•	T	•	•	•	A	A	A	G	C	C	T	•	•	•	G	•	•	•	•	•	•	•	G

Note: • it means conserved sequence

The Table 3 shows the divergent sequence between Genus Tor. *Tor duoronensis* are 0.9-2.16 (mean; 1.53 ± 0.63%); while *Tor tambraides* are 0.93-2.13 (mean; 1.53 ± 0.6). Divergent sequence between *Tor duoronensis* and *Tor soro* was 2-3.7 (mean; 2.65 ± 0.85), while sequence divergent between *Tor duoronensis* and *Tor tambraides* was 2-3.7 (mean; 2, 65-0.85). The high genetic distance indicated a mutation has occurred. This can be observed from different nucleotide bases between samples. The greater of genetic distance between the samples, then the similarity of the nucleotide base is getting smaller. These findings were supported by Mikkelsen et al. (2007) stating for pairwise intraspecific genetic distances range from 0-1.9 percent, while pairwise interspecific genetic distances range from 14-77 percent. The genetic distance for samples with reference related species indicated possible effect of geographical isolation on a population (Mikkelsen et al., 2007). This study correlated with Yang et al. (2013) which stated rapidly emerging evidence of genetic distances without readily observable phenotypic change during the evolutionary history.

Table 3. The Percentage of Genetic Diversity

Species	SKRG	TABR	TORD	TORS	TORT
SKRG	0.3				
TABR	1.15±0.48	0.8±0.4			
TORD	1.53±0.63	1.53±0.6	0.3±0.2		
TORS	2.65±0.85	2.63±0.85	2.58±0,83	1.5±0.6	
TORT	3.25±0.93	2.85±0.85	3.1±0.9	2.43±0.75	1.3±0.6

As a comparison, this study also aligned *Tor tambraides* and *Tor duoronensis* gene sequences obtained from the gene bank and Nguyen et al. (2006) research. A total of 13 sequences of 16S rRNA

genes (9 *Tor duoronensis* species and 4 *Tor tambraides* species) were show the results of Nguyen et al. (Esa & Abdul Rahim, 2013; Nguyen et al. 2008). The current study found that there was a low genetic diversity among species (intraspecies) that is *Tor duoronensis* and *Tor tambraides*, but it had a high diversity to be able of distinguishing this species (*Tor duoronensis* separated clusters with *Tor tambraides*) (Nguyen et al., 2008). The genetic diversity of Genus Tor (*Tor duoronensis* and *Tor tambraides*) might also be affected by environmental factors, including temperature, salinity, and irradiation stress, with significant contributions to mitochondrial sequence variation and mutation rate of this mitochondrial marker (Wallace & Chalkia, 2013).

Similar studies have also been reported by Maralit et al. (2013) which proved that "Pigek" and "Bulidao" fish in the Philippines were the same species namely *Mesopristes cancellatus* which was shown from the results of phylogenetic tree topology based on the 16S rRNA gene sequence that they formed the same clade with a genetic distance of 0.004% between samples. Nugroho et al. (2015) also reported that the intraspecies genetic distance of Nome fish from North Kalimantan had a genetic distance between 0% -0.60%, one genus 0.8-3.24% in a family of 5.10% -14.5%. Genetic relationship among the fish species was examined using a phylogenetic tree. A Phylogenetic tree was made based on the alignment results between the sample and the reference species and comparison with references from (Nguyen et al., 2008). The taxonomic position of *Tor duoronenis* from Pasuruan with *Tor duoronensis* from Padang showed that SKRG-1 and SKRG-2 and TABR-1 and TABR-2 are in one cluster with *Tor duoronensis* (TORD-1 and TORD-2) from Padang. *Tor tambraides* (TORT-1 and TORT-2) and *Tor soro* (TORS-1 and TORS-2) form separate clusters (Figure 2). The phylogenetic tree was made based on ML and NJ methods with the Kimura-2 Parameter and MP calculation model. The selection of Kimura-2 parameter algorithmic calculation models is because it is effective for DNA Barcoding analysis or considering transition and transversion substitution points (Maralit et al., 2013; Juniar et al., 2021; Sari et al., 2021; Priyono et al., 2018). The topologies showed identical and consistent which it was indicated that Tor fish from Pasuruan are *Tor duoronensis*.

The results of phylogenetic topology between TORD from Banyu Biru Lake with the reference species and Nguyen et al. (2006) are that this species is in one cluster with the *Tor tambraides* group, while *Tor soro* and *Tor tambraides* are one cluster with the *Tor duoronensis* group as shown in Figure 3. The topology is supported by a moderate bootstrap value (60/60) using the Kimura-2 Parameter calculation model. This grouping becomes a big question mark because there are differences distinguishing of morphological characters between TORD species with *Tor duoronensis* and *Tor tambraides* from Nguyen et al. (2006). The consistency index is (0.52745), the retention index is (0.7453245), and the composite index is 0.532489 (0.467420) for all sites and parsimony-informative sites in parentheses by Tamura et al. (2013).

The construction of phylogenetic topology was made based on the ML and NJ method with the kimura-2 parameter calculation model. Two methods showed consistency and identical, the slight difference lies in the bootstrap value. The resulting 16S rRNA gene topology was similar to Nguyen's et al. (2006) which produced two main branching groups with moderate bootstrap values. In this study the main branching point is in the bootstrap value of 50-60%. The first cluster is *Tor tambraides* group with *Tor soro*. The second cluster was the *Tor duoronensis* group with samples of TORD from Banyu Biru Lake and *Tor duoronensis*. The low bootstrap value due to short-compared gene sequences, but the specific areas of 16S rRNA gene sequence are able to determine different species (Nguyen et al., 2006; Maralit et al., 2012).

Table 4. The Percentage of Genetic Distance using 16S rRNA Gene

Sample	SKRG-1	SKRG-2	TABR-1	TABR-2	TORD-1	TORD-2	TORS-1	TORS-2	TORT-1	TORT-2
SKRG-1	0									
SKRG-2	0.3									
TABR-1	2	1								
TABR-2	1.3	0.3	0.8							
TORD-1	1.8	1	0.2	1.3						
TORD-2	2	1.3	1.8	1	0.3					
TORS-1	2.3	2.1	2.8	2.8	2.1	2.3				
TORS-2	3.9	3.1	3.4	3.1	3.1	2.8	1.5			
TORT-1	4.4	3.4	3.9	3.9	3.9	3.6	2.8	3.4		
TORT-2	3.1	2.1	2.6	2.6	2.6	2.4	2.5	2.5	2.3	0

Based on 16S rRNA gene included consistency of phylogenetic topology produced, it can be concluded that TORD from Banyu Biru Lake are included in *Tor duoronensis* species. This is consistent with the description of *Torduoronensis* morphology (Haryono & Tjakrawidjaja, 2006; Rahayu et al., 2016; Weber & Beaufor, 1916). TORD from Banyu Biru Lake which have lobes that do not reach the corners of the mouth and can be moved. This is supported by divergent sequences of SKRG (1 and 2) with *Tor duoronensis* at (1-2.13%) (on average, 1.58 ± 0.55%); whereas TABR (1 and 2) has genetic distance with *Tor duoronensis* (1.95 - 2.95%) (average, 2.45 ± 0.5%). SKRG similarity value and TABR with reference species does not show homology with reference gene. SKRG, TABR and *Tor duoronensis* has a similarity value of 97.8% - 99.7% as shown in Table 4. The reconstruction of this phylogeny tree shows that the Sengkaring freshwater found in Banyu Biru Lake are *Tor duoronensis* species which have the same origin as Tor duoronensis from the origin species.

Further diagnostic character of *Tor tambraides* by Nguyen et al. (2006) stated that it has short lobes and do not reach the corners of the mouth, whereas *Tor duoronensis* has long lobes and reaches the corners of the mouth. After searching there was no strong basis used as a reference for determining the taxonomic status of the Tor fish in Sarawak, Malaysia. This is different from the description of Weber and Beaufort (1916), Bleeker (1858), and Haryono & Tjakrawidjaja, 2006 which stated that *Tor duoronensis* has short lobes and does not reach the corners of the mouth, whereas *Tor tambraides* has long lobes and reaches the corners of the mouth. *Tor tambraides* from Nguyen's research similar to *Tor duoronensis* from Indonesia. The distribution of *Tor tambroides* widely and has been reported to occur in Mekong, basins, Peninsular Malaysia (Eschmeyer et al., 2016). Roberts (1999) examined previous collections of *T. tambroides* from Pahang, Johor and Terengganu in Peninsular Malaysia, Bintulu in Sarawak and indicated that fish with long median lobes could be tentatively identified as *T. tambroides*. Sequences reported herein are from specimens that have a long median lobe, and include samples from Pahang river system (TTA06 and TTA09), indicating that *T. tambroides* is a valid species.

The closely related of SKRG and TABR fish from Banyu Biru Lake, Pasuruan with *Tor duoronensis* from Padang (West Sumatra) can be connected with the existence of ancient rivers around 17,000 to 20,000 years ago in the Pleistocene Era. This ancient river flow connects between West Sumatra and East Java. The existence of this ancient river flow allows fish to swim towards the river flow connected to the ancient river to another location. This can be traced from the history of Java, Sumatra, and Kalimantan, which was formerly the area of the Great Sunda Exposure. Separation of Java and Sumatra occurred around the middle of the Miocene when the polar ice melted, sea level dropped, and large areas of the Sunda shelf were exposed and appeared above the surface of the water in the form of vast swampland.

Sea level rise when polar ice waves melted as much as 14.6 to 14.3 kbp which raised sea levels as high as 16 meters in a period of 300 years (Voris, 2000). This separation is believed to be due to the movement of the Earth's plate, the eruption of Mount Krakatau and the fluctuation of sea water. The results of this study indicated that there are variations of *Tor duoronensis* species from Pasuruan with *Tor duoronensis* from Padang (West Sumatra), due to isolation in two different places that have been going on for years.

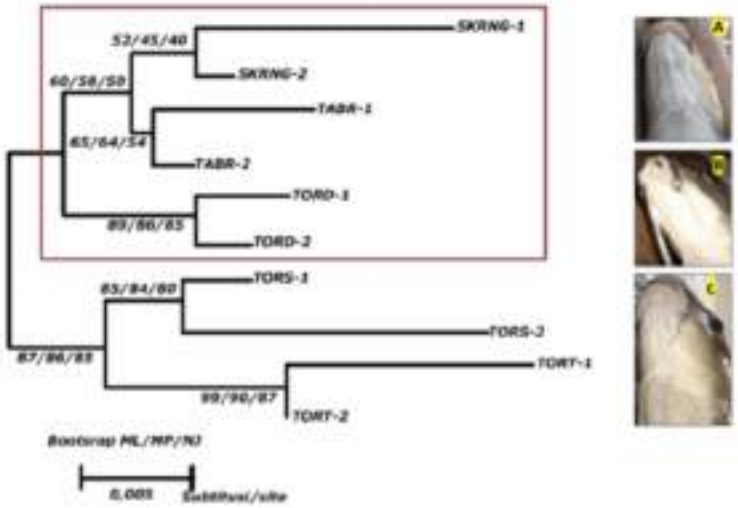

Figure 2. Phylogenetic Tree of SKRG and TABR Based on 16S rRNA Gene. A. SKRG, B. TABR, C. *Tor duoronensis*. Information: SKRG-1=Sengkaring 1; SKRG-2=Sengkaring-2; TABR-1=Tambra-1; TABR-2= Tambra-2; TORS-1=*Tor soro*-1; TORS-2=*Tor soro*-2; TORT-1=*Tor tambraides* 1; dan TORT-2= *Tor tambraides*-2

The closely related of SKRG and TABR fish from Banyu Biru Lake, Pasuruan with *Tor duoronensis* from Padang (West Sumatra) can be connected with the existence of ancient rivers around 17,000 to 20,000 years ago in the Pleistocene Era. This ancient river flow connects between West Sumatra and East Java. The existence of this ancient river flow allows fish to swim towards the river flow connected to the ancient river to another location. This can be traced from the history of Java, Sumatra, and Kalimantan, which was formerly the area of the Great Sunda Exposure. Separation of Java and Sumatra occurred around the middle of the Miocene when the polar ice melted, sea level dropped, and large areas of the Sunda shelf were exposed and appeared above the surface of the water in the form of vast swampland. Sea level rise when polar ice waves melted as much as 14.6 to 14.3 kbp which raised sea levels as high as 16 meters in a period of 300 years (Voris, 2000). The results of this study indicated that there are variations of *Tor duoronensis* species from Pasuruan with *Tor duoronensis* from Padang (West Sumatra), due to isolation in two different places that have been going on for years. If the same rate of mutation is applied, most speciation events among the genus Tor were estimated to occur in the late Miocene or early Pleistocene (6.5–1.25 Mya). It is interesting studied that two contrasting phylogeographical patterns were observed in two sympatrically distributed species, *T. douronensis* and *T. tambroides*. Data from the present study of Nguyen et al. (2006) and Nguyen et al. (2008) revealed high levels of genetic variation or polymorphic site. The exposure of the Sunda Shelf, the extended continental shelf that connects the islands of Sumatra and Borneo to the Southeast Asian mainland, in the Pleistocene epoch (1.6–0.1 Mya) by Voris (2000), is thought to have influenced on altering the river

courses in Southeast Asia, and therefore played an important role in dispersal ability and expansion range of many freshwater species. Present data indicate that the geographically isolated lineages of *T. douronensis* separated in late Miocene and as such the Sunda Shelf seems to have not mediated migration of these lineages between the Borneo and Sumatra islands, and the mainland Southeast Asia.

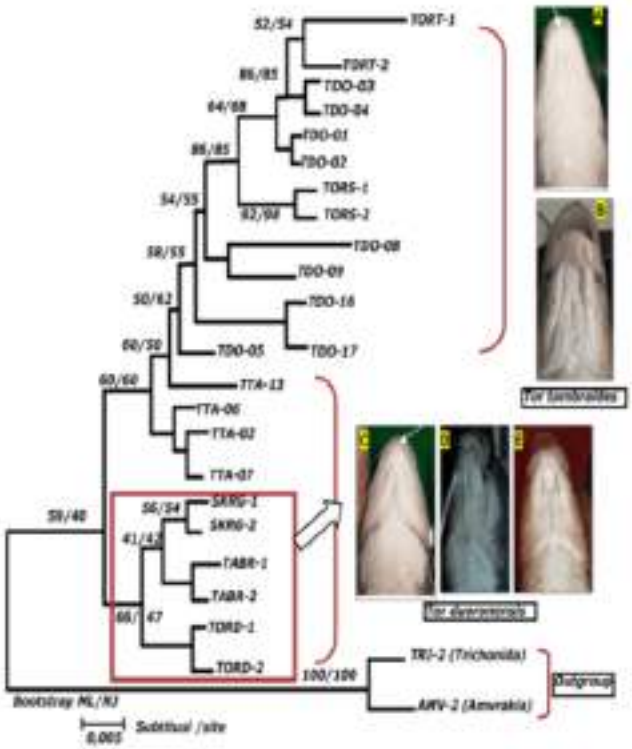

Figure 3. Phylogenetic Tree Based on *Maximum Likelihood* (Bootstrap 1000)

CONCLUSION

This is the first attempt to reconstruct a robust phylogeny of the Genus Tor from Indonesia. When comparing methods of phylogenetic reconstruction and modeling data by 16S rRNA mtDNA gene regions produced identical results to analyses with a three-model applied to the entire using Maximum Parsimony, Maximum Likelihood and Neighbour Joining. The findings from Genus Tor from Pasuruan are closely related to Tor duoronensis from Padang with a bootstrap value of 66%. Sequence was compared and multiple sequence alignment has revealed some polymorphic sites.

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REFERENCES

Bleeker, Petter. (1858). Ichthyologiae archipelagi indici prodromus. Siluri, 382 p. https://www.worldcat.org/title/ichthyologiae-archipelagi-indici-prodromus/oclc/4176897

Dudu, A., Georgescu, S. E., Popa, O., Dinischiotu, A., & Costache, M. (2011). Mitochondrial 16S and 12S rRNA sequence analysis in four salmonid species from romania. *Acta Zoologica Academiae Scientiarum Hungaricae*, *57*(3), 233–246. https://www.sid.ir/en/journal/ViewPaper.aspx?ID=406663

Eprilurahman, R., Simarmata, A. K., Hakim, L., & Trijoko, T. (2021). Morphological and molecular characters of Cherax quadricarinatus (von Martens, 1868) from Sermo Reservoir and Tambakboyo Retention Basin, Daerah Istimewa Yogyakarta, Indonesia. *Biogenesis: Jurnal Ilmiah Biologi*, 9(1), 18. https://doi.org/10.24252/bio.v9i1.16612

Esa, Y., & Abdul Rahim, K. A. (2013). Genetic structure and preliminary findings of cryptic diversity of the Malaysian mahseer (Tor tambroides valenciennes: Cyprinidae) inferred from mitochondrial DNA and microsatellite analyses. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/170980

Eschmeyer, W., Fricke, R., Laan, R. Van Der, & Utrecht, H. (2016). *Catalog of Fishes : Genera , Species , References*. *June*. https://www.calacademy.org/scientists/projects/eschmeyers-catalog-of-fishes

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology*, *3*(5), 294–299. https://doi.org/10.1071/ZO9660275

Glon, M. G., Thoma, R. F., Taylor, C. A., Daly, M., & Freudenstein, J. V. (2018). Molecular phylogenetic analysis of the devil crayfish group, with elevation of Lacunicambarus Hobbs, 1969 to generic rank and a redescription of the devil crayfish, Lacunicambarus diogenes (Girard, 1852) comb. Nov. (Decapoda: Astacoidea: Cambaridae). *Journal of Crustacean Biology*, *38*(5), 600–613. https://doi.org/10.1093/jcbiol/ruy057

Haryono & Tjakrawidjaja, A. . (2006). Morphological Study for Identification Improvement of Tambra Fish (Tor spp.: Cyprinidae) from Indonesia. *Biodiversitas Journal of Biological Diversity*, *7*(1), 59–62. https://doi.org/10.13057/biodiv/d070115

Juniar, A. E., Ambarwati, R., & Rahayu, D. A. (2021). Genetic identification of clithon oualaniense (Gastropoda: Neritidae) from Madura, Indonesia. *AACL Bioflux*, *14*(2), 1046–1056. http://www.bioflux.com.ro/docs/2021.1046-1056.pdf

Kottelat, M., & Whitten, A. J. (1994). Freshwater Fishes of Western Indonesia and Sulawesi. *Copeia*, *1994*(3), 830. https://doi.org/10.2307/1447208

Listyorini, D., Winaris, N., Prananingrum, P., Kartikasari, N., Rahayu, D. A., Khasna, E. N., & Kahrisma, V. D. (2011). *Biologi Molekuler*. https://erlangga.co.id/

Maralit, B. A., Aguila, R. D., Ventolero, M. F. H., Perez, S. K. L., Willette, D. A., & Santos, M. D. (2013). Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety. *Food Control*, *33*(1), 119–125. https://doi.org/10.1016/j.foodcont.2013.02.018

Martins, S., Simões, F., Mendonça, D., Matos, J., Silva, A. P., & Carnide, V. (2013). Median-joining networks for inferring intraspecific phylogenies. *Genetic resources and crop evolution*, *60*(4), 37–48. https://doi.org/10.1007/978-94-009-1207-6_2

Mikkelsen, N. T., Schander, C., & Willassen, E. (2007). Local scale DNA barcoding of bivalves (Mollusca): A case study. *Zoologica Scripta*, *36*(5), 455–463. https://doi.org/10.1111/j.1463-6409.2006.00289.x

Nguyen, T. T. T., Ingram, B., Sungan, S., Gooley, G., Sim, S. Y., Tinggi, D., & De Silva, S. S. (2006). Mitochondrial DNA diversity of broodstock of two indigenous mahseer species, Tor tambroides and T. douronensis (Cyprinidae) cultured in Sarawak, Malaysia. *Aquaculture*, *253*(1–4), 259–269. https://doi.org/10.1016/j.aquaculture.2005.09.014

Nguyen, T. T. T., Na-Nakorn, U., Sukmanomon, S., & ZiMing, C. (2008). A study on phylogeny and biogeography of mahseer species (Pisces: Cyprinidae) using sequences of three mitochondrial DNA gene regions. *Molecular Phylogenetics and Evolution*, *48*(3), 1223–1231. https://doi.org/10.1016/j.ympev.2008.01.006

Nugroho, ED & Rahayu, D. (2015). Status taksonomi ikan nomei dari perairan tarakan, kalimantan utara berdasarkan gen. *Jurnal Harpodon Borneo*, 8(2002), 132–141. https://doi.org/10.35334/harpodon.v8i2.134

Popa, L. O., Popa, O. P., Gargarea, P., & Murariu, D. (2007). Sequence analysis of the 5' COI gene region from Dama dama (Linnaeus, 1758) (Mammalia : Cervidae). *Travaux du Museum National d'Histoire Naturelle Grigore Antipa*, *50*, 537–542. https://eurekamag.com/research/021/737/021737344.php

Priyono, D. S., Solihin, D. D., Farajallah, A., & Arini, D. I. D. (2018). Anoa, dwarf buffalo from sulawesi, indonesia: Identification based on dna barcode. *Biodiversitas*, *19*(6), 1985–1992. https://doi.org/10.13057/biodiv/d190602

Rahayu, DA., Nugroho, ED., Azrianingsih, R., Kurniawan, N., H. (2016). Morphometric Analysis of Local Fish from Banyu Biru Lake, Pasuruan Compared with Closely Related to Tor spp. from Indonesia. *Journal of Tropical Life Science*, *3*(3), 156–159. https://doi.org/10.11594/jtls.03.03.03

Rahayu, DA & Nugroho, E. (2014). Pendekatan Fenetik Taksonomi Dalam Identifikasi Kekerabatan Dan Pengelompokkan Ikan Genus Tor Di Indonesia. *Bioedukasi*, *7*(1), 60–64. https://jurnal.uns.ac.id/bioedukasi/article/view/2844

Rahayu, D. A., & Jannah, M. (2019). *DNA Barcode Hewan dan Tumbuhan Indonesia*. *65*, 1–20. https://osf.io/ty7e5/

Roberts, T. R. (1999). Fishes of the cyprinid genus Tor in the Nam Theun watershed (Mekong Basin) of Laos, with description of a new species. *Raffles Bulletin of Zoology*, *47*(1), 225–236. https://research.si.edu/publication-details/?id=73115

Rozas, J. R. and R. (1995). DnaSP, DNA sequence polymorphism: *Computer Applications in the Biosciences*, *11*(September), 621–625. https://europepmc.org/article/med/8808578

Sari, S. Y. P., Ambarwati, R., & Rahayu, D. A. (2021). Molecular characteristics of Donax faba (Bivalvia: Donacidae) from Nepa Beach, Madura, based on cytochrome oxidase subunit I gene sequences. *AACL Bioflux*, *14*(4), 2416–2426. http://bioflux.com.ro/

Sharma, U., Gupta, D. P., & Mohanty, P. S. (2014). Phylogenetic analysis among Cyprinidae family using 16SrRNA. *International Journal of Fisheries and Aquatic Studies*, *1*(6), 66–71. https://www.fisheriesjournal.com/

Skuza, L., Keszka, S., Panicz, R., & Smietana, P. (2016). Molecular characterization of the noble crayfish (Astacus astacus L.) population from Pomeranian lakes (north-western Poland) based on mitochondrial DNA. *Knowledge and Management of Aquatic Ecosystems*, *2016-Janua*(417), 4–9. https://doi.org/10.1051/kmae/2015046

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Voris, H. K. (2000). Maps of Pleistocene sea levels in Southeast Asia: Shorelines, river systems and time durations. *Journal of Biogeography*, *27*(5), 1153–1167. https://doi.org/10.1046/j.1365-2699.2000.00489.x

Wallace, D. C., & Chalkia, D. (2013). Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. *Cold Spring Harbor perspectives in medicine*, *3*(10), 1–47. https://pubmed.ncbi.nlm.nih.gov/24186072/

Weber, M., & Beaufor, L. F. De. (1916). *The fishes of the Indo-Australian Archipelago*. Brill, Leiden, The Netherlands. https://www.biodiversitylibrary.org/item/46027#page/13/mode/1up

Wong, E. H. K., & Hanner, R. H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, *41*(8), 828–837. https://doi.org/10.1016/j.foodres.2008.07.005

Yang, S., Lai, X., Sheng, G., & Wang, S. (2013). Deep genetic divergence within a "living fossil" brachiopod Lingula anatina. *Journal of Paleontology*, *87*(5), 902–908. https://doi.org/10.1666/12-127

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