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DNA Barcoding of *Neolissochilus sumatranus* and *Tor douronensis* to Support *In Situ* Conservation of Indonesian Mahseers

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ABSTRACT

The fishes of genera *Neolissochilus* and *Tor* are locally known as Ikan Batak or ihan in North Sumatra. They are sacred and are usually served in Batak tribe traditional ceremonies. However, their population in the wild is declining because of habitat degradation, unwise exploitation, water pollution, and the introduction of invasive species. Therefore, suitable management is needed to maintain Ikan Batak's sustainability in their natural habitat, which can be conducted through *in situ* conservation. The habitat of Ikan Batak in the Bonan Dolok river of the Samosir Regency can potentially be developed as a conservation site. Information concerning this species in Bonan Dolok River is needed as a basis for effective *in situ* conservation management. Morphologically identified fish captured in Bonan Dolok River were validated using DNA barcoding of cytochrome oxidase I (COI) mitochondrial DNA segment. This study confirmed that Ikan Batak captured from Bonan Dolok River belongs to two genera, *Neolissochilus* and *Tor*. Furthermore, the phylogenetic tree construction using Maximum Likelihood showed that the specimens identified as *N. sumatranus* were identical to *N.* cf *soroides*, a species found in the Malaysian peninsula, Thailand, and Cambodia.

1. Introduction

Ikan batak or Ihan is a local name for the sacred fish served by the Batak tribe in their traditional ceremonies. Furthermore, the Ikan batak are classified under the two genera *Neolissochilus* and *Tor* in North Sumatra (Larashati *et al.* 2020). In the world, the two genera are known as mahseers and they are widely distributed in the inland waters of southern and southeastern Asia (Rainboth 1985). Geographically, Indonesian mahseers are distributed in Sumatra, Java, and Borneo (Kottelat *et al.* 1993) with different local names depending on their regions. These species are known to have high cultural, economic, and nutritional values.

Four and three species of *Neolissochilus* (*N. thienemanni*, *N. sumatranus*, *N. soro*, and *N. longipinnis*) and *Tor* (*T. tambra*, *T. tambroides*, and *T. douronensis*) are inhabiting Indonesian freshwater (Kottelat *et al.* 1993; Kottelat 2013). Their population has declined because of overexploitation, water pollution, habitat degradation, and introduction of invasive species (Kottelat *et al.* 1993; Haryono and Subagja 2008). *Neolissochilus thienemanni*, is endemic to Lake Toba and has been classified as a vulnerable species by the International Union for Conservation of Nature (World Conservation Monitoring Centre 1996). In addition, it is on the list of Indonesian protected species according to Regulation of the Minister of Environment and Forestry no. 20 of 2018.

One of the efforts to maintain the sustainability of the Indonesian mahseers in the wild is through the development of conservation areas. Based on a previous study, a river at Bonan Dolok Village in Samosir Regency North Sumatra was selected as a prospective conservation area (Larashati and Ridwansyah 2017). A bioecological study was conducted to investigate the potential of the Bonan Dolok River for conservation site of Ikan batak (Larashati *et al.* 2020). Ikan batak from Bonan Dolok River were collected and identified as *N. sumatranus* and *T. douronensis.*

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However, the identification of Ikan batak samples was only based on the morphology. Several studies showed that there had been confusion on the taxonomy of species or between genera of *Neolissochilus* and *Tor* because their morphological features are slightly the same (Laskar *et al.* 2013; Hoàng *et al.* 2015; Walton *et al.* 2017). Moreover, Kottelat *et al.* (1993) mentioned that the taxonomy of the Indonesian Mahseers is confusing and unstable. Therefore, it is important to validate the mahseers using morphological and genetic analysis.

Mitochondrial DNA segments, such as cytochrome oxidase c subunit I (COI) and cytochrome b (cyt b) have been used as DNA barcodes for the identification of some species, including fish. Furthermore, genomic-based identification using mtDNA has been extensively applied to study mahseers phylogenetic and resolve the taxonomic problems in Indian, Malaysian, and Vietnam mahseers (Nguyen *et al.* 2008; Laskar *et al.* 2013; Sati *et al.* 2013; Walton *et al.* 2017). In Indonesia, there have been very few studies conducted on genetic analysis of the mahseers and mostly in *Tor* (Wibowo *et al.* 2013; Wibowo and Kaban 2015).

The cornerstone of conservation relies on a solid taxonomic base and an understanding of how animals can be grouped into coherent management units. We subsequently initiated a study to validate the Ikan batak species composition from our previous research (Larashati *et al.* 2020) using molecular analysis of the COI mitochondrial DNA segment. Our study can be used as biological information of the mahseers in North Sumatra inland waters and it has implication for the conservation of Ikan batak.

2. Materials and Methods

2.1. Sample Collection

Ten fish specimens were collected from Bonan Dolok River, Samosir Regency, North Sumatra (Figure 1), and nine were morphologically identified as *N. sumatranus* and one as *T. douronensis* (Larashati *et al.* 2020). The fishes were identified based on Weber and Beaufort (1916), Kottelat *et al.* (1993), and Kottelat (2013). In the previous study, muscle tissues or fin have been cut from specimen's right side and preserved in 96% ethanol. Afterwards, the specimens were fixated in 4% formaldehyde.

2.2. Molecular Work and Analysis

To further confirm the taxonomy position of samples obtained and their phylogenetic relationship, DNA was extracted from the collected muscle or fin part following the DNA purification of mammalian tissue and rodent tail protocol from GeneJet Thermo Fisher Scientific. The concentration and purity of extracted DNA were measured using NanoDrop™ One Microvolume UV-Vis Spectrophotometer. COI DNA segments were amplified and sequenced using forward primer 5'TCAACCAACCACAAAGACATTGGCAC3' and reverse primer 5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward *et al.* 2005). Amplification was conducted using PCR Thermal Cycler (Biorad) with PCR condition as followed (Steinke and Hanner 2011): 2 min at 95°C, 35 cycles of 0.5 min at 94°C, 0.5 minutes at 52°C, and 1 minute at 72°C, 10 min at 72°C, held at 4°C. The PCR products were visualized in 1% agarose gel containing SYBR safe. The purification and bidirectional sequencing were performed by 1st BASE DNA Sequencing Services (Malaysia).

The sequences of the samples were edited and entered into similarity search for species identification using Basic Local Alignment Search Tool (BLAST) (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi). According to Bhattacharjee *et al.* (2012), similarity ranges of 97–100%, 92–96%, and ≤91% between the sample and the database sequence are categorized as significant, moderate, and insignificant, respectively. The multiple sequences obtained from samples and NCBI were aligned by ClustalW using MEGA version x (Kumar *et al.* 2018). The same program was used to construct Maximum Likelihood Tree using two cyprinids *Barbodes binotatus* (accession number: MG699688.1) and *Barbonymus gonionotus* (accession number: KU692343.1) as outgroup species. Furthermore, phylogenetic confidence was estimated by bootstrapping with 1000 replicate data sets. The pairwise genetic distance between populations was calculated using the Tamura-Nei distance implemented in MEGA.

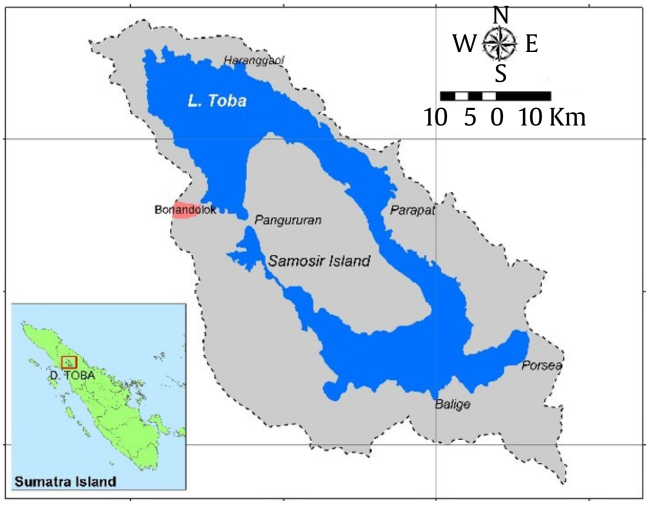

Figure 1. Sampling location in Bonan Dolok River, Samosir Regency, North Sumatra (Larashati and Ridwansyah 2017)

3. Results

3.1. Morphological Characteristics of Ikan Batak Samples

Based on the morphological characterization, the BD2 sample showed a lack of tubercles on the cheek and a median lobe (Figure 2), demonstrating that BD2 is a *Tor*. Another characteristic, such as the length of the stiff part of the last simple dorsal ray, is similar to the length of the head without a snout which indicated that BD2 is *T. douronensis*. Other morphological features were pectoral fin is longer than the dorsal fin, 3 bony and 8 weak dorsal rays, 3 bony and 5 weak anal rays, 1 bony and 16 weak pectoral rays, 1 bony and 8 weak ventral rays, 8 predorsal scales, 25 lateral line scales, and 12 caudal peduncle scales.

The collected specimens of *N. sumatranus* exhibited a lack of median lobe and the presence of tubercles (Figure 2). In some, the length of the stiff part of the last simple dorsal ray was shorter than that of the head without a snout (BD3, BD4, BD6, BD8, BD9, and BD10), and others showed the same length (BD1, BD5, and BD7). The pectoral fin of some

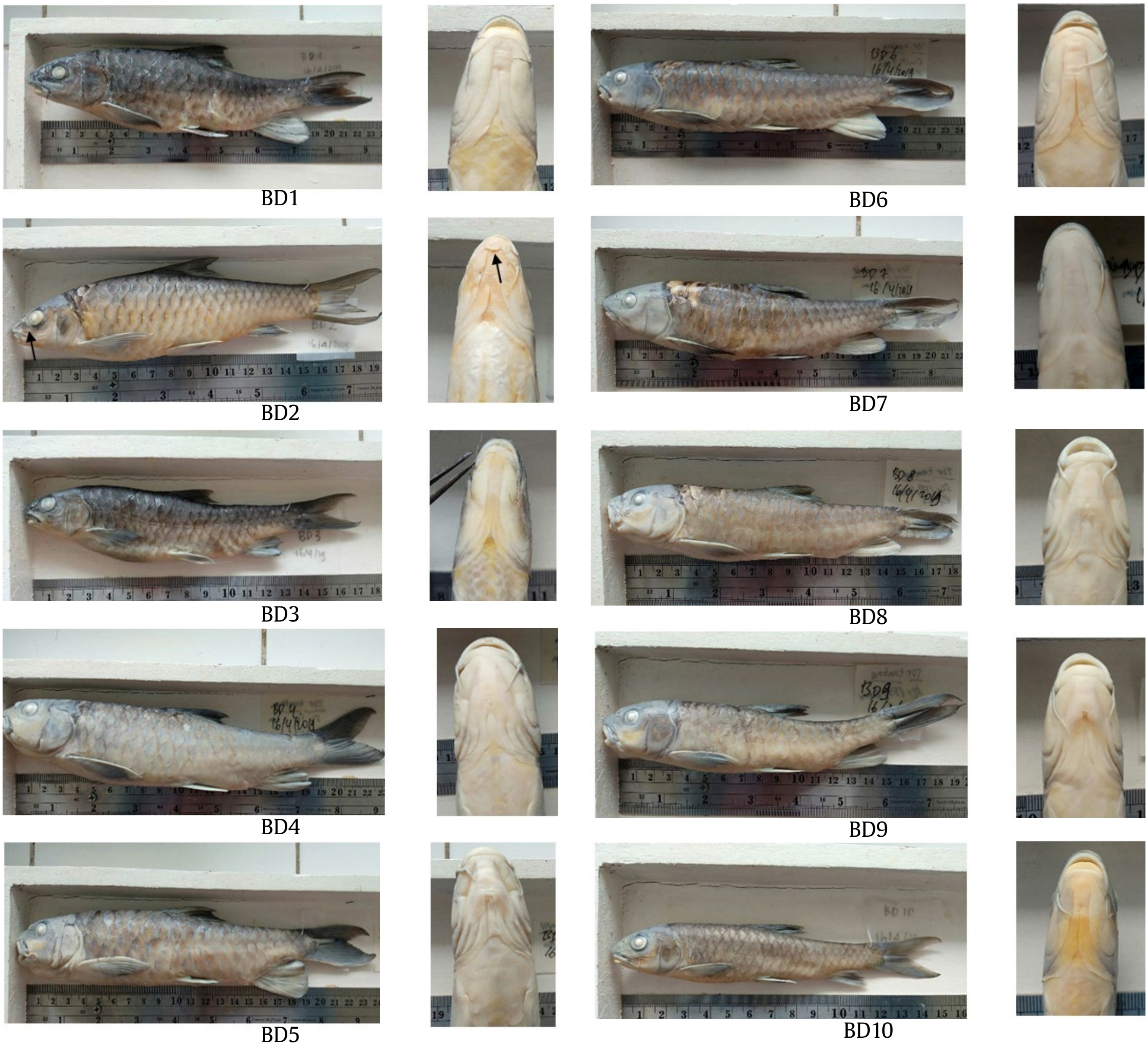
BD1 BD6 BD2 BD7 BD3 BD8 BD4 BD9 BD5 BD10

Figure 2. Ikan batak samples from Bonan Dolok River (*N. sumatranus*: BD1, BD3-10, *T. douronensis*: BD2). The arrow in BD2 sample shows the median lobe

specimens was longer than (BD1, BD5, and BD7) or the same as (BD3, BD4, BD6, BD8, BD9, BD10) the dorsal. Other morphological features were 3 bony and 8–9 weak dorsal rays, 2–3 bony and 5–6 weak anal rays, 1 bony and 14–16 weak pectoral rays, 1 bony and 8 weak ventral rays, 8 predorsal scales, 24–27 lateral line scales, and 12 caudal peduncle scales.

3.2. Molecular Identification and Phylogenetic Relationship of Ikan Batak Samples

The mtDNA COI gene of approximately 650 bp length was amplified and sequenced from ten Ikan batak samples. A similarity search in the Genebank database showed that the *N. sumatranus* samples were significantly similar (99.71%) to *N. soroides* (accession number: AP011314), while *T. douronensis* sample was significantly similar to *T. tambra* (accession number: KT354857) (97.86%) and to *T. douronensis* (accession number: KP712053) (97.13%). The genetic distance of *T. douronensis* sample and *T. douronensis* in the Genebank database was 0.035 (Table 1). This indicated different species following the study conducted by Ward *et al.* (2009), where a 3% difference in DNA barcode of fishes indicated that the samples are different species. The genetic distance of *N. sumatranus* samples and *N. soroides* was 0.002, which indicated that *N. sumatranus* samples and *N. soroides* were the same species. The genetic distance of *N. sumatranus* samples and *N. soroides* was 0.002, which indicated that *N. sumatranus* samples and *N. soroides* were the same species. The collected specimens of Bonan Dolok River, clustered in two different groups are also shown in the phylogenetic tree (Figure 3). Sample BD2 belong to *Tor*, and other samples belong to *Neolissochilus*. *Neolissochilus sumatranus* samples were clustered together with *N. soroides*.

4. Discussion

The phylogenetic analysis is consistent with the morphological identification, which indicated that the samples are divided into *Neolissochilus* and *Tor*. The *N. sumatranus* samples were identical to *N. soroides*, a species distributed in Thailand, Cambodia, and Malaysia (Rainboth 1991). The BD2 sample is closely related to *T. douronensis*, a species inhabiting the inland waters of Sundaland and has been collected from the rivers in some Indonesian regions, including Batang Toru-South Tapanuli and Asahan River in North Sumatra (Lumbantoruan *et al.* 2013; Roesma and Chornelia 2016). In this study, the main character to distinguish *Neolissochilus* and *Tor*, the presence or absence of a median lobe described by Rainboth (1985), can be applied.

Findings from the present study also confirm the synonymity of *N. sumatranus* and *N. soroides* as mentioned by Roberts and Khaironizam (2008). The authors compared *N. soroides* specimens from Peninsular Malaysia to *N. sumatranus* from West Sumatra stored in the Zoological Reference Collection of the National University of Singapore, where both species were reported to be similar. It was tentatively concluded that *N. sumatranus* is a junior synonym of *N. soroides*. Some of its morphological features, such as the type of lower lip, lateral line scales, predorsal scales, and caudal peduncle scales, meet that of *N. soroides* (Khaironizam *et al.* 2015). *Neolissochilus sumatranus* lives only in Sumatra's inland waters (Kottelat *et al.* 1993) and has been collected from the rivers in North Sumatra (Simanjuntak 2012; Barus *et*

Table 1. Genetic distances between species of samples and Genbank database

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
N. sumatranus BD1, BD3-10														
T. douronensis BD2	0.048													
T. tor	0.049	0.033												
T. putitora	0.050	0.031	0.017											
T. khudree	0.050	0.031	0.032	0.022										
T. douronensis	0.056	0.035	0.041	0.036	0.036									
T. tambroides	0.052	0.033	0.034	0.027	0.031	0.038								
T. barakae	0.043	0.031	0.026	0.023	0.026	0.026	0.031							
Neolissochilus sp.	0.035	0.039	0.036	0.033	0.033	0.044	0.041	0.030						
N. stracheyi	0.027	0.046	0.043	0.047	0.044	0.059	0.050	0.037	0.032					
N. cf soroides	0.002	0.046	0.047	0.048	0.048	0.054	0.050	0.041	0.033	0.025				
T. tambra	0.048	0.022	0.027	0.020	0.020	0.035	0.027	0.023	0.031	0.046	0.046			
N. soroides	0.002	0.046	0.047	0.048	0.048	0.054	0.050	0.041	0.033	0.025	0.000	0.046		
Barbonymus gonionotus	0.142	0.154	0.149	0.153	0.149	0.158	0.143	0.156	0.142	0.140	0.140	0.151	0.140	
Barbodes binotatus	0.188	0.196	0.184	0.184	0.193	0.197	0.181	0.187	0.187	0.181	0.191	0.193	0.191	0.185

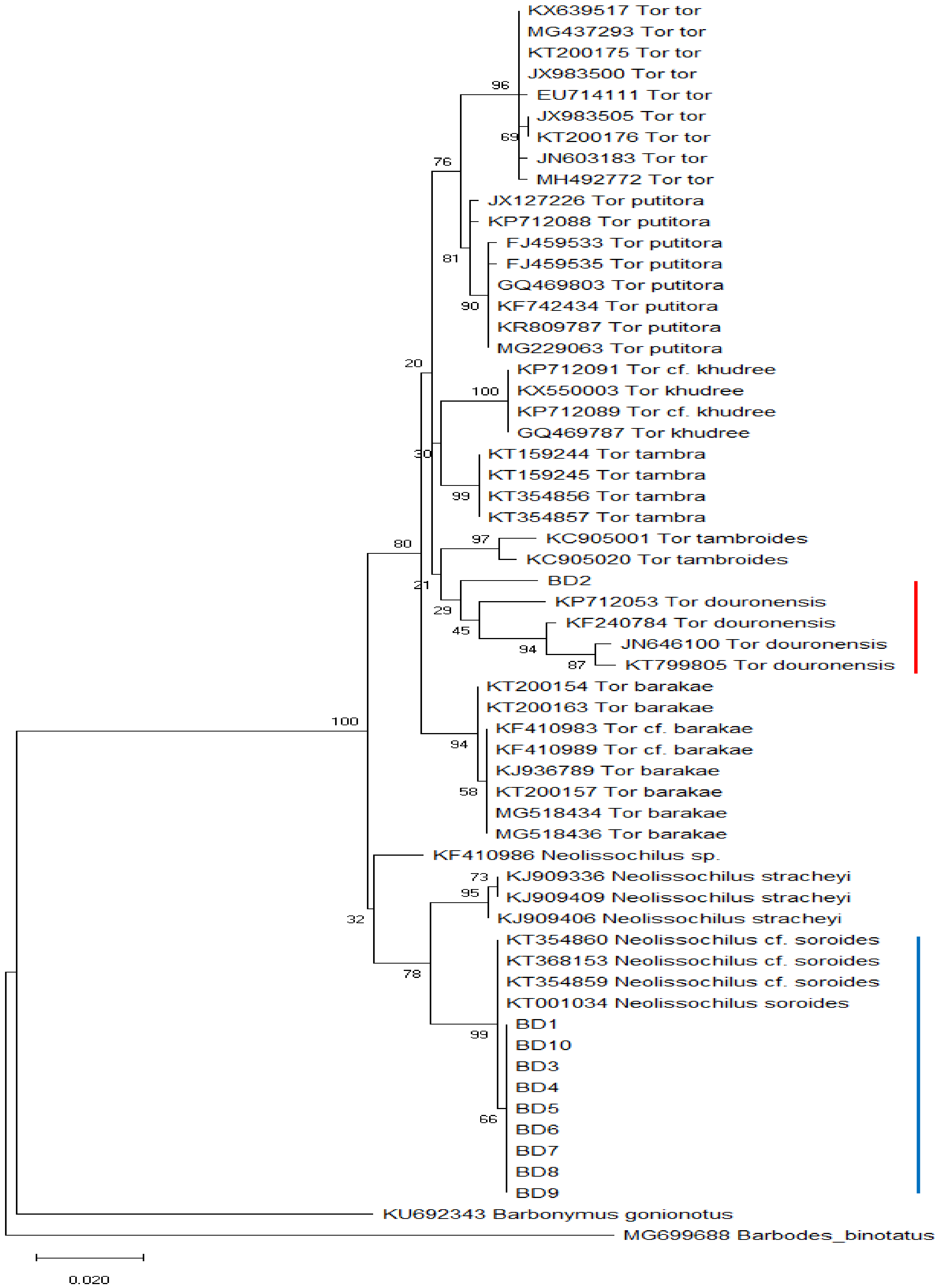

Figure 3. Maximum likelihood tree of ikan batak samples based on the COI mitochondrial gene sequences with bootstrap value of 1,000 replicates

al. 2014; Larashati and Widoretno 2016; Roesma *et al.* 2016). Our finding suggested that the distribution location of *N. sumatranus*, which is only in Sumatra as mentioned by Kottelat *et al.* (1993), should be revised. *Neolissochilus soroides* collected from the upper Gombak River of Peninsular Malaysia showed some variations in the mentum (a fleshy median lobe between the lower lips) (Roberts and Khaironizam 2008). Some of *N. soroides* exhibit thick and long mentum, while others have the lips like the true *Neolissochilus* and truncated type. Meanwhile, the variation within the species is called intraspecific polymorphism, and the collected specimens from Bonan Dolok River did not have the median lobe in their lower lips except for a sample identified as Tor (BD2). However, a complete study with more samples is needed to understand the intraspecific polymorphism between *N. sumatranus* samples since some of the collected specimens showed truncated type.

Mahseers conservation has been impeded because of the taxonomy ambiguity across the two genera, Tor and *Neolissochilus* (Pinder *et al.* 2019). The median lobe cannot always be used to differentiate the *Neolissochilus* and *Tor* since there are variations in the mouth structure within the genus *Neolissochilus* and *Tor* (Roberts and Khaironizam 2008; Walton *et al.* 2017). Combining the molecular data may elucidate the taxonomic relationship. The COI gene has been used to validate the taxonomic position of Indian mahseers confirming that *N. hexastichus* is a valid species and *T. progeneius* is a synonym of *T. putitora* and suggesting the conservationist to focus on *T. putitora* conservation (Laskar *et al.* 2013). Furthermore, the systematics status of *T. tambroides* and *T. douronensis* from the regions in Malaysia has been confirmed using the COI gene (Esa *et al.* 2008). In this study, the genetic distance of BD2 and *T. tambra* shows that they are the same species. However, the phylogenetic tree shows that BD2 is clustered with *T. douronensis* even though the genetic distance shows different species. The phylogenetic tree shows *T. douronensis* from three localities (North Sumatra: BD2, Sabah: Accession number KF240784, KT799805, JN646100, and Yunnan: Accession number KP712053), indicating that different haplotypes are generated from the localities which can be used as a unique or distinguishing character. Further study is needed to determine and analyse the haplotypes useful for in situ conservation efforts.

Other species within the genus *Neolissochilus, N. soro*, have also been collected from some rivers in North Sumatra. The fish was previously mentioned as *T. soro* (Kottelat 2013). In Indonesia, it is the only mahseer that has been released as an aquaculture fish (Gustiano *et al.* 2013; Subagja and Juli 2014). Furthermore, the fish is very popular and considered sacred in other regions besides North Sumatra, such as in West Java. The fish is tentatively regarded as a junior synonym of *T. tambra*, but the lack of mental lobe has placed it in *Neolissochilus* (Roberts 1999; Kottelat 2013). There has not been any genetic study to confirm the fish as *Neolissochilus*. Considering that intraspecific polymorphism exists in some species (Roberts and Khaironizam 2008; Hoàng *et al.* 2015) and the similar appearances of *N. sumatranus* and *N. soro*, it raises a question whether they belong to similar species. A genetic study of *Neolissochilus* should be conducted to confirm their taxonomic status.

N. sumatranus and *T. douronensis* are currently listed as Not Evaluated in the IUCN. Taxonomic ambiguity for the two species may be the cause for not been thoroughly assessed. Pinder *et al.* (2019) and Kottelat *et al.* (2013) stated that *T. douronensis* is similar to *T. tambra*. However, morphology-based identification of Indonesian mahseers is still based on the study of Kottelat *et al.* (1993), where *T. douronensis* was described as a valid species. Furthermore, Kottelat *et al.* (2018) suggested to categorize *T. tambra* as Data Deficient until the taxonomy of the *T. tambra* is reevaluated. *Neolissochilus soroides* is listed as Least Concern with the description that the level of threat to this species is still unknown and some healthy species are still found in protected areas (Ahmad and Vidthayanon 2019).

The population of Indonesian mahseer in the wild are rapidly declining. Therefore, conservation and proper management of the mahseers are needed for their sustainability in the natural habitat. A valid identity of Indonesian mahseers through morphology and molecular based methods is needed to recognize their conservation status and develop the conservation or management strategy. An example of fisheries management strategy is through population enhancement by stocking (Kartamihardja 2015). Information on the fish diversity, especially native species living in the water body, is important to prevent non-native species stocking.

Bonan Dolok River, which stretches 1 km in a hilly area, provides a suitable habitat for the mahseers, potentially to be developed as a conservation site (Larashati *et al.* 2020). Protecting the mahseers means conserving the whole biodiversity and the habitats since the mahseers need clean flowing water and high dissolved oxygen (Khajuria and Langer 2016). This study's preliminary information showed that two genera of mahseers, *Neolissochilus* and *Tor*, which consists of *N. sumatranus* and *T. douronensis*, are inhabiting Bonan Dolok River as part of the native fish species, where *N. sumatranus* is identical to *N. soroides.* Further study needs to be conducted with more sampling sites along Bonan Dolok River to fully describe the species composition and abundance of Ikan batak in Bonan Dolok River and their habitat preferences as well as the analysis on the genetic diversity of *N. sumatranus*.

Conflicts of Interest

The authors confirm that there are no potential financial and competing interests related to this manuscript that need to be stated.

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