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Genetic differentiation among Batak fish populations (*Neolissochilus sumatranus*, *Tor douronensis*, and *Tor soro*) in North Sumatra, Indonesia revealed by RAPD markers

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Abstract. *Barus TA, Wahyuningsih H, Simanjuntak BN, Ginting RH, Batubara AS, Hartanto A. 2023. Genetic differentiation among Batak fish populations (*Neolissochilus sumatranus, Tor douronensis, *and* Tor soro*) in North Sumatra, Indonesia was revealed by RAPD markers. Biodiversitas 24: 1327-1332*. Mahseer or Batak fish species within the genera of *Neolissochilus* and *Tor* are highly valued as a source of food for local communities, especially in North Sumatra. Assessment of genetic differentiation of the Batak fish population, namely Tor soro, Tor douronensis, and Neolissochilus sumatranus in the North Sumatra Rivers, has been conducted. The objective of this study was to determine the possible genetic divergence among congeneric fish and supply the genetic information for the design of conservation strategies. Fish specimens were collected from three rivers in North Sumatra Bahorok River (Langkat District), Asahan River (Toba Samosir District), and Batangtoru River (South Tapanuli District). The genetic analysis employed the Random Amplified Polymorphism DNA (RAPD) markers using a set of RAPD primers: OPA-2, OPA-3, OPA-6, OPA-7, OPA-9, OPA-11, and OPA-17. The amplified fragments were analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) 2.00 program to construct the dendrogram of relationship among accessions. The genetic similarity coefficient among species of *T. soro*, *T. douronensis*, and *N. sumatranus* reached 0.44-0.86, with the lowest similarity at 44%. The cluster analysis indicated that *T. douronensis* is more closely related to *N. sumatranus* than *T. soro* based on the grouping of accessions. There is an indication of natural hybrids occurrence between *N. sumatranus* and *T. soro* populations despite the different habitats and locations of sampling. Our study revealed that using RAPD markers may discriminate inter- and intraspecific Batak fish populations in North Sumatra.

Keywords: Conservation, dendrogram, fish specimens, mahseer, population genetics

INTRODUCTION

Mahseer (common name) or *ikan* (*ihan*) *Batak/ jurung* (vernacular name) is sacred fish species commonly consumed in a traditional ceremony by the Bataknese or Batak tribe, which inhabit the warm climate of Indonesian waters and consists of two genera, namely *Neolissochilus* and *Tor* (Larashati et al. 2020). Neolissochilus members comprise four species, *Neolissochilus thienemanni, N. soro, N. sumatranus,* and *N. longipinnis* (Kottelat et al. 1993; Kottelat 2013). Based on the species record, one *Neolissochilus* species, namely *N. sumatranus,* was reported to reside in Asahan River, North Sumatra (Weber and de Beaufort 1916; Purba et al. 2013; Barus et al. 2014). The locals called this species *jurung batu* or *ikan batak* by the Bataknese tribe. The species has been regarded as an endemic biological resource in the freshwater of North Sumatra, especially in some rivers surrounding the Asahan River and Lake Toba (Dinoto et al. 2020; Larashati et al. 2020).

Based on published records, 24 species of *Neolissochilus* have been identified worldwide (Kottelat 2013; Hoang et al. 2015; Khaironizam et al. 2015). However, some species have experienced a significant decline in population due to intensive fishing and destructive fishing gear (Chaudhry and Barbhuiya 2010; Kottelat 2020; Vidthayanon 2012; Ali et al. 2014; Manimekalan et al. 2015; Sahoo et al. 2015; Ho and Ahmad 2019). This phenomenon also occurs in *N. sumatranus* native population, implying that conservation efforts are required to protect them as important biological resources in North Sumatra (Prianto et al. 2017). Furthermore, several studies have been reported, such as the analysis of the morphology of *N. sumatranus* in Batang Toru, North Sumatra (Roesma et al. 2019); the culturable gut bacteria of *N. sumatranus* in Toba Samosir, North Sumatra (Dinoto et al. 2020). While research on genetic characterization and differentiation is rarely reported, especially for North Sumatran accessions (Wibowo et al. 2013; Wibowo and Kaban 2015). Therefore, this research is important data for the conservation plan of endangered native fish species in North Sumatra.

In less than a half-century, molecular markers have completely altered our perception of population divergence, and they have evolved. RAPD markers, derived from PCR amplification of genomic DNA, are a crucial tool in evolutionary biology (Ali et al. 2004).

The existence of different fish individuals with different phenotypes may result in a mixed genetic composition in a

population and ecosystem (Batubara et al. 2018; Yulianto et al. 2020; Batubara et al. 2021). Genomic profiling using RAPD barcode analysis is a simple and popular method for analyzing genetic diversity in different populations. RAPD analysis can provide genetic diversity, genetic distance, and a relationship dendrogram for various fish populations and varieties. It has been reported that the method is practical for fish genetic variety mapping (Danish et al. 2012; Ikpeme et al. 2015; Neekhra et al. 2014).

Genetic diversity, also known as genetic variability, is crucial for the well-being of individuals within a population and the population's survival as a whole. This diversity allows for adaptation to changing environmental conditions and helps populations to withstand environmental stressors, including thermal disturbances, pollution, and diseases (Nati et al. 2021). Batak fish conservation efforts have been carried out through a series of laboratory studies to increase their stocks (Kamarudin et al. 2012). These thoughts emphasize a crucial component in the genetic conservation of a particular species population. The technique of releasing farmed fish into the wild is common. Still, without careful genetic study, it may jeopardize preservation aims by homogenizing fish populations and lowering species diversity. Furthermore, it is critical to recognize that various conditions necessitate different responses. As a result, the goal of conservation efforts should be to create an integrated approach that conserves as much genetic variation within species as feasible while still ensuring the availability of utilizable fish resources (Almeida et al. 2013). There is a marked decline in the mahseer population across various ecosystems, threatening its survival (Roesma et al. 2017). Degradation of genetic diversity, caused by factors such as shrinking population size or habitat destruction, reduces a species' ability to adapt and increases the risk of extinction. Hence, it is crucial to determine the genetic variation among and within mahseer populations in North Sumatra and to identify populations with a high genetic variation that can be utilized as germplasm for aquaculture and conservation purposes.

MATERIALS AND METHODS

Study period

This research was conducted in 2019. First, fish specimens were collected in three rivers, i.e., Bahorok River (Langkat District: 3°29'16.2" N, 99°11'7.1" E), Asahan River (Toba Samosir District: 2°33'21.8" N, 99°18'43.5" E) and Batangtoru River (South Tapanuli District: 1°28'42.9" N, 99°3'30.2" E). The collected fish samples were then transported to the Integrated and Medical Laboratory, Universitas Sumatera Utara, Indonesia, for experimentation (Figure 1).

Sampling procedure

Sampling locations were selected based on the information from local fishermen who may have discovered an abundance of Batak fish. Sampling was conducted from 08.00 am to 4.00 pm. Fish samples were caught using a casting net (size 1.5 inches), gill net (size 1.5 and 2 inches), and fish hooks.

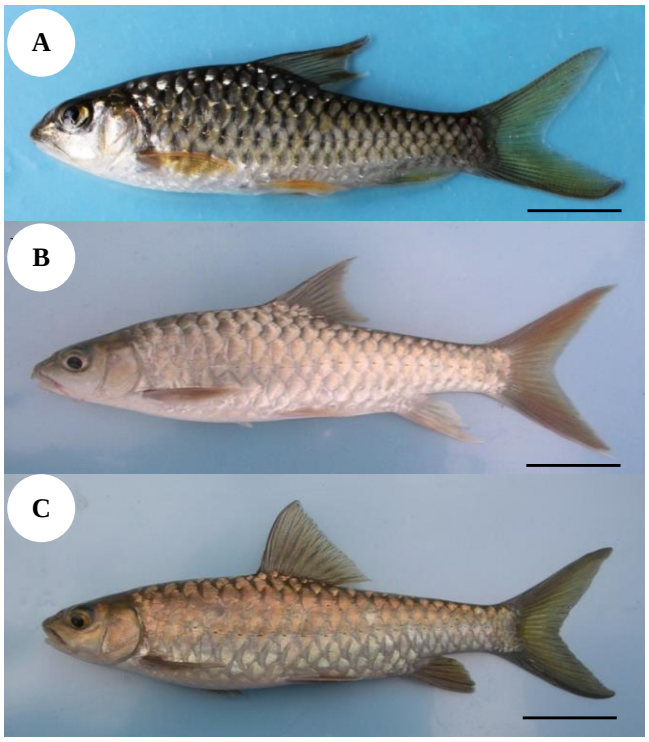

Figure 1. Batak fish species in the North Sumatran Waters, i.e:A. *Neolissochilus sumatranus*, B. *Tor douronensis* and C. *Tor soro*. Scale bar: 3 cm

Next, using a digital caliper, the captured specimens were measured for their total and standard length (Sigma Digital, 500-196. Error = 0.01 mm). Next, the body mass was weighed using a digital scale (Lesindo, LS-03. Error = 0.01 g). The specimens were photographed for field documentation, labeled for their location and time of catching then euthanized using clove oil. The fish samples were frozen in an ice box (Marina Cooler 10 L) and transported to the laboratory for identification. The identification step followed the Truss method used in fish morphometrics, which is the study of the shape and size of fish. The Truss method quantifies fish morphological shape by constructing a truss network that describes the relationships among morphological landmarks on a fish body (Strauss and Bookstein 1982). Species identification using key morphological traits was compared with an identification guidebook by Haryono et al. (2020).

DNA extraction

A total of five fish samples were randomly selected based on species and then photographed separately. Approximately 1 cm of pectoral fin tissue was taken from each specimen using sterile scissors to avoid contamination. The fin tissue was put into a 2.0 mL microtube which had previously been added with 96% alcohol, then labeled and taken to the laboratory. At the same time, the fish samples were preserved in a 10% (v/v) formaldehyde solution. Fish genomic DNA was extracted following the tissue genomic DNA mini kit protocol with minor modifications. First, a piece of dorsal fin tissue (± 30 mg) was inserted into a 1.5 mL tube and crushed using a

grinder, following the addition of 200 µL GT buffer and homogenized. Next, to the sample, 20 µL of proteinase K solution was added, homogenized, and incubated at 60ºC for 30 min. After incubation, a 200 µL GT buffer GT was added, homogenized for 5 s, and re-incubated for 20 min at 60ºC. The top layer of solution was to a new 1.5 mL tube and diluted with 200 µL absolute EtOH. The solution was placed into the GS column and centrifuged at 13,000 rpm for 2 min. Next, the pellets were collected, added with 400 µL W1 buffer, and centrifuged at 13,000 rpm for 30 s. Next, the pellets were transferred into a new 2 mL tube added with 600 µL washing buffer and centrifuged at 13,000 rpm for 30 s. Finally, the pellets were transferred to a new collection tube and centrifuged further at 13,000 rpm for 3 min to dry the column matrix. Next, the dried column was transferred into a sterile 1.5 mL tube, added with 100 µL pre-heated elution buffer, and let to absorb for 5 min. Next, the solution was centrifuged at 13,000 rpm for 30 s to obtain the pure DNA.

DNA amplification

DNA amplification used MyTaq HS Red Mix I with a set of RAPD primers OPA-2, OPA-3, OPA-6, OPA-7, OPA-9, OPA-11 and OPA-17 (Roesma et al. 2017, Sharma et al. 2016). The PCR program was run in a thermal cycler with the specification: pre-denaturation at 95ºC for 2 min, denaturation at 95ºC for 45 sec, annealing with different optimum temperatures of 36ºC (OPA-2), 37ºC (OPA-3), 40ºC (OPA-6, OPA-7, OPA-11, and OPA-17), 390C (OPA 9) for 1 min, elongation at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR reaction was performed for 30 cycles. The PCR results were visualized on an agarose gel electrophoresis for 60 min at 100 V. Gel was stained in 1 µg/mL of EtBr solution for 15 min, then washed with distilled water for 10 min. The RAPD fragments were observed in a UV transilluminator (G.BOX SYNGENE) and documented.

Data analysis

The RAPD fragments were scored as presence (1) and absence (0) of DNA bands to generate a binary data set from each population. Then, the genetic analysis utilized the binary data set as a similarity coefficient to construct a dendrogram of relationships using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) performed in NTSYS-pc version 2.00.

RESULTS AND DISCUSSION

Amplification using RAPD primers was carried out on *Neolissochilus sumatranus*, *Tor douronensis*, and *Tor soro* populations, resulting in scorable RAPD fragments from 7 primers (Figures 2, 3, 4, and 5). The number of DNA bands produced from each individual varied depending on the RAPD primers used in this study. The visualized DNA bands successfully amplify the randomly attached primer in fish genomes. The number of DNA bands produced by each primer depends on the distribution of homologous sites with the primary sequence in the genome. In addition, the formation of DNA band fragments depends on the primer sequence and genotype of the DNA template. Several primers did not produce any scorable bands, which indicated a non-compatibility with DNA templates (Suresh et al. 2013). In addition, the varying intensity of amplicons can also be caused by competing primers attaching to similar sites on the DNA template or the absence of amplified sites due to non-compatible primers to the DNA template (Ganaie and Ali 2014).

The genetic relationship of the Batak fish can be evaluated based on the genetic similarity between the species. RAPD results may be converted into a binary data set retrieved from the 15 accessions to construct a dendrogram of kinship (Figure 6). The genetic similarity among fish species ranged from 0.44 to 0.86, with the lowest similarity percentage at 44% (Table 1). Generally, each population forms adjacent clusters based on the RAPD score or genetic distances with a slight overlap in some accessions. The dendrogram showed two main clusters that appeared to be separated at a genetic distance of about 0.69, which distinguished the majority of TS (*Tor soro*) accessions from TD (*Tor douronensis*) and NS (*Neolissochilus sumatranus*).

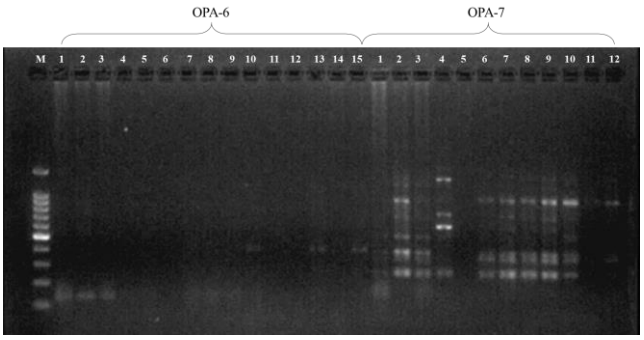

Figure 2. RAPD profile using OPA-6, OPA-7, M= Marker 1.5 kb, Lane 1-5 = *Tor soro*, 6-10 = *Tor douronensis*, 11-15 = *Neolissochilus sumatranus*

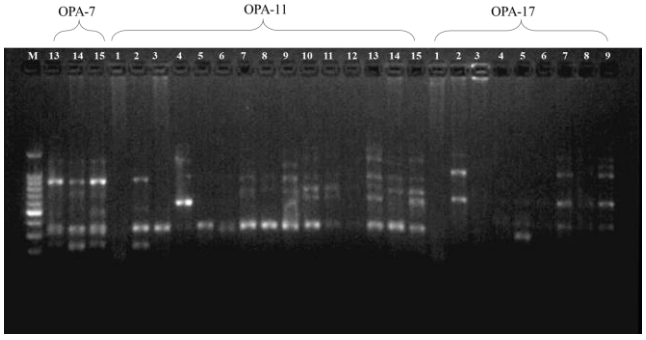

Figure 3. RAPD profile using OPA-7, OPA-11, OPA-17 M= Marker 1.5 kb, Lane 1-5 = *Tor soro*, 6-10 = *Tor douronensis*, 11-15 = *Neolissochilus sumatranus*

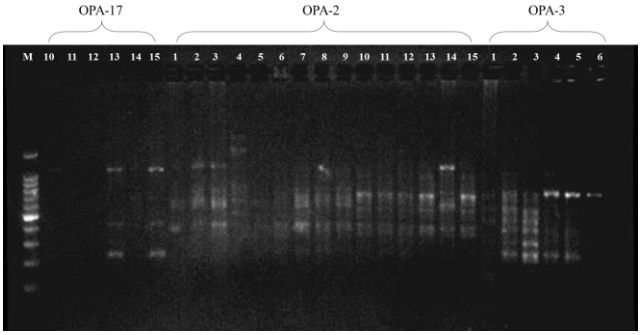

Figure 4. RAPD profile using OPA-17, OPA-2, OPA-3, M= Marker 1.5 kb, Lane 1-5 = *Tor soro*, 6-10 = *Tor douronensis* , 11-15 = *Neolissochilus sumatranus*

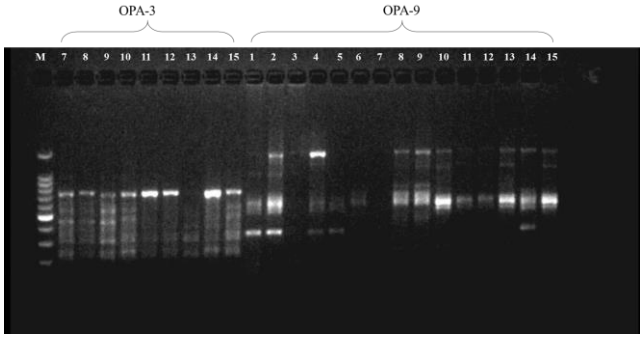

Figure 5. RAPD profile using OPA-3, OPA-9, M= Marker 1.5 kb, Lane 1-5 = *Tor soro*, 6-10 = *Tor douronensis* , 11-15 = *Neolissochilus sumatranus*

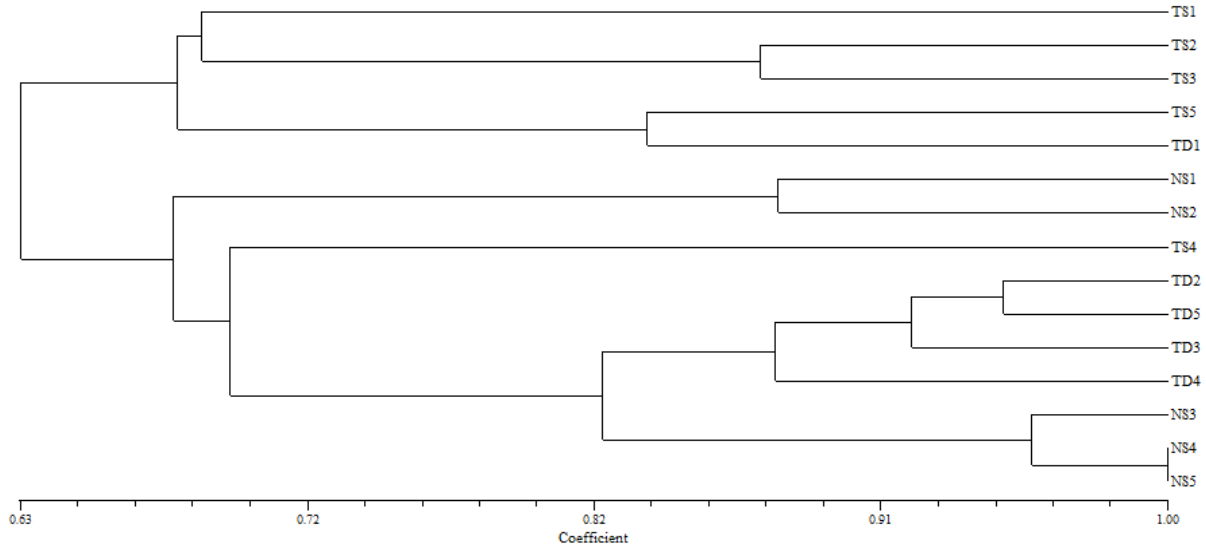

Figure 6. UPGMA dendrogram shows the genetic relationship among all accessions *Tor soro* (TS), *Tor douronensis* (TD), and *Neolissochilus sumatranus* (NS)

Table 1. Genetic similarity of *Neolissochilus sumatranus* (NS), *Tor douronensis* (TD) and *Tor soro* (TS)

Accessions	TS1	TS2	TS3	TS4	TS5	TD1	TD2	TD3	TD4	TD5	NS1	NS2	NS3	NS4	NS5
TS1	1														
TS2	0.631	1													
TS3	0.750	0.869	1												
TS4	0.533	0.727	0.631	1											
TS5	0.666	0.631	0.750	0.533	1										
TD1	0.666	0.631	0.750	0.533	0.833	1									
TD2	0.533	0.636	0.631	0.666	0.666	0.800	1								
TD3	0.533	0.636	0.631	0.666	0.666	0.666	0.800	1							
TD4	0.444	0.800	0.636	0.666	0.555	0.666	0.857	0.857	1						
TD5	0.500	0.695	0.600	0.736	0.625	0.750	0.947	0.947	0.909	1					
NS1	0.666	0.636	0.631	0.666	0.666	0.666	0.777	0.666	0.666	0.736	1				
NS2	0.615	0.500	0.588	0.500	0.615	0.615	0.625	0.625	0.526	0.588	0.875	1			
NS3	0.555	0.800	0.727	0.761	0.555	0.666	0.761	0.761	0.833	0.818	0.857	0.7336	1		
NS4	0.47	0.75	0.666	0.7	0.588	0.705	0.8	0.8	0.869	0.857	0.800	0.666	0.956	1	
NS5	0.470	0.75	0.666	0.7	0.588	0.705	0.8	0.8	0.869	0.857	0.800	0.666	0.956	0.99	1

The Batak fish population may be classified into two main clusters, (1) comprised TS1, TS2, TS3, TS5, TD1 at 0.684, (2) comprised NS1, NS2, TD2, TD5, TD3, TD4, NS3, NS4, NS5 accessions at 0.82, while TS4 was placed as an outlier at 0.693. The next cluster that separates NS from TD can be separated by a genetic distance above 0.82. Based on the construction of the dendrogram, there appeared to be other outliers, namely NS (NS1, NS2) and TD1, which are grouped into the TS population. The PCR-RAPD technique has been used to validate the existence of regionally adapted populations within a species that may have emerged either through genetic selection under distinct environmental conditions or due to genetic drift. Ecological, evolutionary, and historical factors can all influence the differentiation of the population genetics of a species.

The similarity coefficient of *T. soro* obtained in this study reached from 0.53 to 0.86 (Table 1), which was higher than previous studies with a similarity coefficient ranging from 0.12 to 0.39, 0.18 to 0.26, and 0.23 to 0.71 (Nugroho et al. 2007; Asih et al. 2008; Simanjuntak et al. 2018). Genetic diversity may indicate gene flow between populations, environmental selection, larval migration patterns, and random allele combinations (Lutz et al. 2022). The large genetic diversity may also indicate good adaptability by each population to thrive in their environment. Genetic variation is an important indicator of a species or population's long-term sustainability and a population's tolerance to adapt, driven by environmental alterations. Species that exhibit both good phenotypic and genotypic diversity are likely to be more fit and capable of surviving in their habitats (Pasztor et al. 2016).

Tor douronensis is closer to *N. sumatranus* than to *T. soro*. The genetic similarity among *N. sumatranus* populations ranged from 0.66 to 0.99 or the lowest similarity percentage at 66%, *T. douronensis* populations ranged from 0.66 to 0.94, the lowest similarity percentage at 66%, and *T. soro* populations ranged from 0.53 to 0.86 the lowest similarity percentage at 53%. The analysis at the species level in the second cluster showed that *T. douronensis* is closer to *N. sumatranus* (different regions) than to *T. soro* population based on its genetic distance. The average similarity value between *N. sumatranus* and *T. douronensis* reached 0.73, while that between *T. soro* reached 0.63 (Table 1). These results may occur due to the similar morphology, physiology, and behavior of *N. sumatranus* and *T. douronensis* to *T. soro* at the allele level. In addition, it might also be due to the habitat differences affecting the genetic composition of certain fish individuals (Nevado et al. 2013; Wellband et al. 2018). There can be significant morphometric and genetic differences between populations if there is enough isolation within a small geographic area because there is no gene flow. On the contrary, a study by Asih et al. (2008) which distinguished different *T. soro* accessions from North Sumatra and West Java, reported no genetic differences among populations based on RAPD profiles. Simanjuntak et al. (2018) reported the possibility of new variants of *T. soro* from Batang Toru streams with a genetic distance of 0.23-0.71.

One of the factors that may initiate a genetic drift in multiple habitats is isolation from distant regions, eventually leading to more obvious genetic differences. Each habitat's biological and ecological factors will also determine the fish's tolerance and survival ability (Nicol et al. 2017). Mutations and migration are two factors that contribute to increased genetic diversity, whereas natural selection and genetic drift events decrease genetic diversity (Gardner et al. 1991). In addition, a close kinship among fish populations from different rivers is likely to have occurred in the past when these rivers were interconnected (ancient rivers) (Tan et al. 2012). To our understanding, the genetic study of *N. sumatranus* is limited. A recent report by Larashati et al. (2022) showed that the existence of *N. sumatranus* in the Sumatran region was still debatable and may be identical to *N.* cf *soroides* based on cytochrome oxidase I (COI). There is a evidence to suggest the occurrence of natural hybridization between *N. sumatranus* and *T. soro* populations, despite their differences in habitat and location of sampling. That highlights the potential for these species to interbreed and form hybrid populations in areas where their habitats overlap. It is important to consider the implications of such hybridization for conserving and managing these species. The study then reveals evidence that RAPD barcodes could be used for genetic differentiation of closely related Batak fish species and their population in the North Sumatran regions.

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