

Taxonomy confirmation of *Puntius cf. binotatus* from Gunung Tujuh Lake, Jambi, Indonesia based on Cytochrome Oxidase-I (COI) gene

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Abstract. Roesma D. I, Tjong D. H, Karlina W, Aidil D. R. 2019. Taxonomy confirmation of *Puntius cf. binotatus* from Gunung Tujuh Lake based on Cytochrome Oxidase-I (COI) gene. *Biodiversitas* 20: 54-60. The population of *Puntius cf. binotatus* Gunung Tujuh Lake in considered as an isolated population which found in the 20-30 meters depth under the water Gunung Tujuh Lake, Sumatra Island. The species often exhibit different phenotypic characters and may genetic characters may also different due to their response to habitat/ecological conditions. Previous study on *P. binotatus* from several locations in West Sumatra showed the complexity of the genetic and morphological characters. A molecular study using COI gene of *P. cf. binotatus* from Gunung Tujuh Lake in Sumatera has been conducted to determine the taxonomic status of the fish. The distinct morphological characters of *Puntius cf. binotatus* is one black spot in the middle of the caudal and the absent of black spot on the base of the anterior dorsal ray. The analysis on COI gene showed that the range of sequence divergences between *P. cf. binotatus* Gunung Tujuh Lake and other Sumatra *P. cf. binotatus* is 3.1-7.6% and 4.1% to *Barbodes banksi*. This value represents the differences at the subspecies level of *Barbodes banksi*. Therefore *Barbodes banksi gunungtjuluh* is proposed as an appropriate name to *P. cf. binotatus* Gunung Tujuh Lake.

Keywords: Morphology, Cyt b gene, COI gene, Gunung Tujuh Lake, *Puntius cf. binotatus*

INTRODUCTION

Gunung Tujuh Lake, Kerinci District, Jambi Province is located in Sumatra Island, Indonesia at an altitude of 1,950 meters above sea level, surrounded by volcanic mountains that have seven peaks. There is no river flow into the lake as the water source for the lake, except for water catchment surrounding that entering the lake through the small rivers. Water flows out of the lake directly forming a 75 meters high waterfall. It is believed that the fish live in this lake have been isolated for a long period. It has been proven that the population in perfectly isolated habitats that prevent the migration of individuals between populations. If there is limited gene flow between populations, the frequency of genes in a population will be different from before, in which the genetic mixing between populations still occur (Roesma et al. 2017). Handika et al. (2014) reported that there is a type of fish with genus *Puntius* in Gunung Tujuh Lake that live in the 20-30 meters depth under the lake. Based on the phenotypic characters identified, the species is similar to *P. binotatus*, so it is identified as *P. cf. binotatus*. According to Champasri et al. (2007) and Collins et al. (2012), Silver Barb or Spotted Barb are the common names of *Puntius*, and they are sold for food and ornamental fish.

Based on molecular studies using the cytochrome b gene (cyt b) mitochondrial DNA, Roesma (2011) and Roesma et al. (2016) reported that *P. binotatus* from several locations in West Sumatra reflected the complex and variable genetic and morphological characters among the locations and considered them as *P. cf. binotatus*. Vitri et al. (2012) reported that *P. binotatus* from six populations

consisting of five rivers (Salibutan, Batang Kuranji, Sungai Asam, Batang Gumanti and Batang Sinamar) and one lake (Lake Maninjau) in West Sumatra showed significant morphological differences among the locations. Recent study, Karlina et al. (2016) using cyt b gene on *P. cf. binotatus* from Gunung Tujuh Lake and *P. cf. Binotatus* recommended that *P. cf. binotatus* from Gunung Tujuh Lake as a candidate of a new species.

According to Kottelat (2013), *Puntius* and *Barbodes* are synonymous. Previously Taki et al. (1978), Mohsin and Ambak (1983), and Rainboth (1996) used the *Barbonymus*, *Barbus*, *Barbodes*, *Systomus*, *Capoeta*, and *Hypsibarbus* as the generic names. Roesma et al. (2018) reported those genera are not synonym and *P. cf. binotatus* is suggested as *Barbodes banksi* from West Sumatra that consist of at least three subspecies based on Cytochrome Oxidase-I (COI) gene. Based on the previous studies, it is crucial to use COI gene to identify and verify the taxonomic status of Silver Barb (*P. cf. binotatus*) from Gunung Tujuh Lake. A mtDNA gene has been widely used for taxonomic, phylogenetic and phylogeography studies in the Silver Barb (John et al. 2013; Singh et al. 2013; Negi et al. 2017). The COI gene is a part of mtDNA used for species identification (DNA barcode). The DNA barcode is designed to develop a rapid species identification that also allows detecting new species (Hebert et al. 2003a; Stoeckle 2003; Hebert and Gregory 2005). COI gene has rapid evolutionary rates than other mtDNA genes. Therefore, is useful for identification at species level, and has been applied to identify numerous animal taxa (Hebert et al. 2003a; Clare et al. 2006; Hajjibabaei et al. 2007; Smith et al. 2008; Frezal and Leblois 2008).

MATERIAL AND METHODS

A total of 50 individuals of *P. cf. binotatus* in Gunung Tujuh Lake, Kerinci District, Jambi Province, Indonesia were collected following Cailiet et al. (1986). A piece of the tissue samples collected from individuals and stored in Eppendorf tubes (1.5 mL) containing 96% ethanol PA. Individual samples were temporarily stored in 10% formalin and transferred in 70% ethanol for long-term storage. Morphological measurements were done for 29 morphometric and 17 meristic characters following Kottelat et al. (1993) and Haryono and Tjakrawidjaja (2006) with additional three characters: Length of upper caudal (LUCL), Length of lower caudal (LLCL) and Length of middle caudal (LMCL). Morphometric and meristic characters were measured using digital calipers to the nearest 0.1 mm.

Total genomic DNA was extracted using Invitrogen PureLink™ Genomic DNA Mini Kit. The result of DNA extraction was used for Polymerase chain reaction (PCR) amplification using thermal cyclers (SensoQuest). PCR was conducted in 25 µl volumes containing 2x MyTaq Hs Red Mix (Bioline), 0.01 mM forward and reverse primer, 35 ng/µl DNA template and 10 µl nuclear-free water. Primer used were *forward* (Fish F1: 5'TCAACCAACCACAAA GACATTGG CAC3') and *reverse* (Fish R1: 5'TAGACTTCTGGGTGGCCAAAGAATCA3') (Ward et al. 2005). The optimal PCR condition consisting of initial denaturation at 95°C for 2 minutes, 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 54°C for

30 seconds, elongation at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were visualized in 2% agarose at 100 Volt, 20 W for 50 min. Good quality PCR product was used as samples for sequencing, performed in MacroGen USA DNA Sequencing Laboratory.

A total of 46 sequences of COI gene were analyzed consisted of four sequences *Puntius cf. binotatus* in Gunung Tujuh Lake, 17 sequences *P. cf. binotatus* of other populations in Sumatra and 25 sequences taken from Genbank NCBI included outgroup species (*Rasbora daniconius*, *Danio choprai*, and *Botia rostrata*) (Table 1). DNA sequences (forward and reverse) were assembled and edited using the DNA STAR program (Burland 2000). DNA sequence of *P. cf. binotatus* compared with the sequences contained in NCBI, to check the similarity of all sequences in <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. All sequences were aligned using the Clustal X program (Thompson 1997). The aligned sequences were edited using BIOEDIT program (Hall 1999). The number of polymorphic sequences was analyzed using the DNA Sequence Polymorphism 5.10 (Rozas 2003). Sequence divergences of all sequences were analyzed using the Kimura-2 parameter model (K2P) in the MEGA 6.0 program (Tamura 2013). The phylogenetic tree was reconstructed based on four methods; Neighbors Joining (NJ), Maximum Evolution (ME), Maximum Likelihood (ML) and Maximum Parsimony (MP) using MEGA 6.0 (Tamura 2013).

Table 1. List of freshwater fish species barcoded along with GenBank Accession Numbers

Family	Genus	Species	Location	Accession No.	Sources
Cyprinidae	<i>Puntius</i>	<i>Puntius binotatus</i>	Lampung	JQ665834	Wibowo et al. (2012)
		<i>Puntius binotatus</i>	Malaysia	JN646096	Sade and Biun (2012)
		<i>Puntius banksi</i>	Malaysia	JF781235	Song et al. (2013)
		<i>Puntius sophore</i>	India	JX983465	Khedkar et al. (2014)
		<i>Puntius denisonii</i>	India	HQ176003	Lakra et al. (2010)
		<i>Puntius orphoides</i>		JF915642	Collins et al. (2011)
		<i>Puntius sarana</i>	India	EU417805	Lakra et al. (2008)
		<i>Puntius sarana</i>	India	JQ667570	Khedkar et al. (2014)
		<i>Puntius sarana subnasutus</i>		HE664122	Vincent (2012)
		<i>Barbodes</i>	<i>Barbodes binotatus</i>	Malaysia	KT001028
		<i>Barbodes binotatus</i>	Thailand	KP263421	Panprommin and Chanto (2014)
	<i>Systemus</i>	<i>Systemus sarana</i>	India	JX983454	Khedkar et al. (2014)
		<i>Systemus sarana</i>	India	KJ476776	Lal et al. (2014)
		<i>Systemus orphoides</i>	Thailand	KP263417	Panprommin and Chanto (2014)
	<i>Barbus</i>	<i>Barbus matthesi</i>	Afrika	KR153301	Sakharova (2015)
		<i>Barbus camptacanthus</i>	Afrika	HM418123	iBOL (2010)
		<i>Barbus callipterus</i>	Afrika	HM418124	iBOL (2010)
		<i>Barbus brazzai</i>	Afrika	KT193523	Decru et al. (2015)
	<i>Barbonymus</i>	<i>Barbonymus schwanefeldii</i>	Malaysia	KT001008	Meganathan et al. (2015)
		<i>Barbonymus schwanefeldii</i>	Laos	JQ346171	Pasco-Viel et al. (2012)
	<i>Barbonymus altus</i>	Afrika	KU568767	Van Der Walt et al. (2012)	
	<i>Barbonymus gonionotus</i>	Philippina	HQ682667	Aquino et al. (2011)	
<i>Rasbora</i>	<i>Rasbora daniconius</i>	India	JX983478	Khedkar et al. (2012)	
	<i>Danio</i>	<i>Danio choprai</i>	Myanmar	KX852477	Kullander and Noren (2016)
Botiidae	<i>Botia</i>	<i>Botia rostrata</i>	China	KM610433	Chen et al. (2015)

RESULT AND DISCUSSION

The morphological characters of *Puntius cf. binotatus* in Gunung Tujuh Lake (Table 2) were similar to *Puntius binotatus* described by Weber and de Beaufort (1916); Roberts (1989 and Kottelat et al. (1993). The characters that distinguish *P. cf. binotatus* in Gunung Tujuh Lake and *P. binotatus* from other area are the absence of a black spot on the base of the anterior dorsal ray and in the part of the body. According to Vitri et al. (2012), ten morphological characters of *P. binotatus* in West Sumatra were different significantly, and also showed the morphological variations at a different altitude.

The total size of the COI gene is 720 bp and 564 bp of them were successfully amplified. The average of nucleotide composition was of A: 27.30%, C: 27.20%, T: 29.40%, and G: 16.10%. The nucleotide composition was similar to previous studies (Negi et al. 2017; Roesma et al. 2018). The frequency of distributions for the four nucleotide bases was significantly different, with non-overlapping confidence intervals. The proportion of pyrimidines (T, C) to purines (A, G) deviated from a 50:50 ratio is well described in the literature for protein-coding genes (Kartavtsev et al. 2009). The bias of the nucleotide bases reflects the hydrophobic properties of the encoded proteins (Nailor et al. 1996). The A + T content (56.70%) in present study higher than G + C (43.30%). Those values indicate the characteristic of the mitochondrial DNA of all classes of fishes (Johns and Avise 1998; Kartavtsev et al. 2007; Kartavtsev et al. 2011; Xiao et al. 2012; Negi et al. 2017).

Of the 564 bp COI gene sequences, 352 bp (62.41%) was conserved sites, and 212 bp (37.59%) was the variable site without insertion or deletion. In the total of 212 variable sites, 183 bp (86.32%) are parsimony informative site, and 29 bp (13.68%) are singleton site. In total, there were 337 mutations in all sequences, the transitions are

more common than transversion, as found in animal mitochondrial genomes (Briolay et al. 1998). The bias value of transition and transversion ratio (Ts; Tv) is R: 7.208. *P. cf. binotatus* from Sumatra showed 71 substitutions which consist of 62 transitions and nine transversions without insertion and deletion. The COI gene is representing 188 amino acids for all samples. Among all of the mutations were observed in *P. cf. binotatus* of Sumatra, change has occurred only in one amino acid that is in the 172nd amino acid (I→V). The calculated nucleotide diversity (π) in the 46 sequences was of 0.11608, Notably, for *P. cf. binotatus* in Sumatra, the nucleotide diversity (π) was 0.04650, that value is at the middle level when compared to the other reported species of *Puntius* (Negi et al. 2017). The value indicated that in Sumatra, there are *P. cf. binotatus* which vary genetically.

Four phylogenetic methods (ME/NJ/ML/MP) were used to construct the phylogenetic tree (Figure 1) The topologies of ML tree was similar to NJ, ME and MP using 1000 bootstrap replicates. Two main clusters were obtained, supported by high bootstrap value of 97/98/95/96 for ME/NJ/ML/MP, respectively. The first cluster consists of five subclusters and the second cluster consists of four subclusters. All the members in the second cluster are the genus that considered as a synonym to *Puntius* (*Barbus*, *Barbonymus*, *Puntius*, and *Systomus*). The sequence divergences that separate the first and second cluster was of 14.2-20.4%. When referring to sequence divergence value reported by Kartavtsev et al. (2007) and Kartavtsev et al. (2011), all the member of the first cluster cannot be considered *Puntius*. The sequence divergences between *P. cf. binotatus* in Gunung Tujuh Lake with other *P. cf. binotatus* in Sumatra were of 3.1-7.4%.

In the first subcluster of the topology, *P. cf. Binotatus* Gunung Tujuh Lake is in the same cluster with *P. cf. binotatus* from Diatas lakes, Batang Lembang, Batang Gumanti, Muara Pingai rivers, and *P. banksi* from Malaysia.

Table 2. Morphometric measurements of *Puntius cf. binotatus* in Gunung Tujuh Lake, Kerinci, Jambi, Indonesia

Characteristics	Code	Range (mm) n=50			%Standar Length (SL)		
		Max	Min	Mean ± SD	Max	Min	Mean ± SD
Total length	TL	80.54	49.2	61.90 ± 7.12	147.24	105.56	129.36 ± 5.28
Standard length	SL	62.83	37.7	47.93 ± 5.96	-	-	-
Head length	HL	18.22	11.2	14.08 ± 1.54	40.02	25.26	29.51 ± 2.35
Head Depth	HD	12.16	6.95	9.73 ± 1.10	30.30	14.44	20.42 ± 2.26
Head Width	HW	10.52	4.87	7.11 ± 1.05	20.55	11.95	14.88 ± 1.69
Eye Diameter	ED	4.15	2.07	2.89 ± 0.34	8.23	4.03	6.09 ± 0.84
Interorbital width	IW	5.96	3.43	4.76 ± 0.58	14.95	7.69	9.99 ± 1.20
Mouth Length	ML	27.1	1.42	2.67 ± 3.54	53.01	2.95	5.55 ± 6.90
Interorbital length	IOL	14.6	8.92	10.94 ± 1.27	25.68	19.01	22.89 ± 1.43
Snout of length	SNL	23.39	2.46	6.24 ± 4.15	54.06	6.02	13.20 ± 9.44
Body Depth	BD	17.08	4.99	9.67 ± 3.72	33.64	10.67	20.14 ± 7.13
Body Width	BW	20.43	4.44	10.81 ± 3.98	34.94	10.85	22.60 ± 8.00
Caudal Peduncle Length	CPL	11.39	6.24	8.78 ± 1.04	29.13	13.42	18.45 ± 2.38
Dorsal Base Length	DBL	8.68	4.12	6.74 ± 0.95	18.40	10.06	14.13 ± 1.77
Dorsal Fin Height	DFH	13.96	7.27	9.96 ± 1.46	32.32	15.62	20.92 ± 3.01
Ventral Fin Length	VFL	10.79	5.1	7.97 ± 1.21	21.30	12.75	16.67 ± 1.95
Anal Fin Length	AFL	9.42	4.97	7.03 ± 0.92	19.03	10.33	14.77 ± 1.87

The sequence divergences between *P. cf. binotatus* Gunung Tujuh Lake and other members in that first subcluster are 3.1-4.1%, supported with the highly bootstrap value (99/99/96/98 for ME/NJ/ML/MP, respectively). Sequence divergences between *P. cf. binotatus* Gunung Tujuh Lake and *P. banksi* from Malaysia was 4.1%. Referring to the values reported by Ward et al. (2005), Kartavtsev et al. (2009), Kartavtsev (2011), and Kartavtsev (2013), this value places *P. cf. binotatus* Gunung Tujuh Lake is at the sub-species level. Based on Cyt b gene, Karlina et al. (2016) reported that the sequence divergences between *P. cf. binotatus* Gunung Tujuh Lake and others population (*P. cf. binotatus* from Diatas lakes, Batang Lembang, Batang Gumanti, Muara Pingai rivers and *P. banksi* from Malaysia) were 6.6-8.1% which positions it as a subspecies. Therefore both types of the genes provide the same conclusion that *P. cf. binotatus* Gunung Tujuh Lake is at the level of sub-species.

Puntius cf. binotatus Gunung Tujuh Lake is a sister taxon of *P. cf. binotatus* from other populations in the first subcluster. This closed relationship may be because of two group of fish live in the same geographical and ecological conditions. All populations of *P. cf. binotatus* in the first subcluster are located at the highland over 1.500 meters above sea level in the eastern part of Bukit Barisan Mountain range (Roesma et al. 2018). Bukit Barisan Mountain range is the row of the mountain that spreading out from northern to southern of Sumatra island more or less 1650 km in length. It is estimated that these conditions cause local adaptations to bring their current genetic variation. Negi et al. (2017) reported that *P. sopherae* from the distinct region with the most homogeneous environment has low genetic diversity compared the populations from the small geographical region with a heterogeneous environment.

The sequence divergences between *Puntius cf. binotatus* Gunung Tujuh Lake and other members in the same subcluster with the member of the second subcluster (*Barbodes binotatus* Thailand with the accession number of KP263421 and *Barbodes binotatus* Malaysia with the accession number of KT001028) are 3.7-4.5%. Consequently, *P. cf. binotatus* Gunung Tujuh Lake and other members in the first subcluster can be grouped in *Barbodes* because of the sequence divergences value do not support them to differ at genus levels. Besides the position of the branch in the tree, this provision also refers to Ward et al. (2005), Kartavtsev et al. (2009), Kartavtsev (2011), and Kartavtsev (2013).

The sequence divergences between the first and second subclusters in the first cluster with the member of the third subcluster (*Puntius cf. binotatus* Batang Kuranji River, *P. cf. binotatus* Batang Tarok River, and *P. cf. binotatus* Batang Tarok River) are 6.3-7.0%. That value indicated that they are different at the subspecies level. Furthermore, the sequence divergences between the first, second, third subclusters in the first cluster with the member of the fourth subcluster are of 6.7-8.5%. Those values also indicated that they are different the subspecies. All of the four subclusters have the sequence divergences value of 11.6-13% to *P. binotatus* Malaysia with the accession

number of JN646096 in the fifth subcluster of the first cluster. Based on the values of Ward et al. (2005), Kartavtsev et al. (2009), Kartavtsev (2011), and Kartavtsev (2013), the range of 11.6-13% value shows that all of the four subclusters differ at the species level to *P. binotatus* Malaysia with the accession number of JN646096.

Puntius binotatus from Malaysia with the accession number JN646096 occupies the position at the cladogram that suggests it as the common ancestor to all of *Puntius* in Sumatra. In NCBI data Genbank, *P. binotatus* Malaysia with the accession number JN646096 and *P. binotatus* Lampung with the accession number JQ665834 listed as *Barbodes binotatus* while in the IUCN Red List the *Barbodes binotatus* stated as the accepted scientific/valid name of *Puntius binotatus* (Eschmeyer 2015; Jenkins et al. 2015; IUCN 2018). In addition, *B. binotatus* Thailand (KP263421), *B. binotatus* Malaysia (KT001028), and *B. binotatus* Lampung (JQ665834) cannot be classified as *P. binotatus*. Hence, all member of the first cluster is considered as the genus of *Barbodes*.

The genetic distance between *Puntius banksi* Malaysia (JF781235) and other members at same branch (*P. cf. binotatus* Batang Gumanti River, *P. cf. binotatus* Diatas Lake, *P. cf. binotatus* Muaro Pingai River, and *P. cf. binotatus* Batang Lembang River) is ranged between 0.9% to 1.3%, thus this group is identified as *B. banksi*. This results of this study implied that there are four subspecies of *B. banksi* in Sumatra. *P. cf. binotatus* Gunung Tujuh Lake is proposing as *B. banksi gunungtjjuh*.

Studies by those of Kartavtsev and Lee (2006); Kartavtsev et al. (2007); Kartavtsev et al. (2009); Kartavtsev (2011); Kartavtsev et al. (2014) reported that the range of sequence divergences values of COI gene between *P. cf. binotatus* Gunung Tujuh and other Sumatra *P. cf. binotatus* was of 3.1-7.6%, indicating differences at the subspecies level. However, a study by Karlina et al. (2016) using the Cyt b gene suggested a sequence divergence value of 6.6-11.2% indicating differences at different species levels. According to (Hebert et al. 2003a; Hebert et al. 2003b) the COI gene is more representative to be used to distinguish species. This support *P. cf. binotatus* Gunung Tujuh as a subspecies (*B. banksi gunungtjjuh*). The second cluster consists of four sub-clusters consisted of genera that synonym of *Puntius* (*Barbus*, *Barbodes*, *Systemus*, and *Barbonymus*). This group is supported by high bootstrap values (98/97/95/97 for ME/NJ/ML/MP respectively). The sequence divergences between genera in the second cluster are 10.7-24.1% and with the first cluster is 13.7-21.1%. These values show that they are different genus but the same family. At the second cluster of each genus that is considered a synonym of *Puntius* clustered on different sub-clusters, there are overlapping of placements in the *Systemus* and *Puntius* groups in the third subclasses. In the IUCN Red List, Fish Base and Catalog of life (Dahanukar 2010; Froese and Pauly 2018; IUCN 2018) the *S. sarana* and *S. orphoides* stated as the accepted scientific/valid name of *P. sarana* and *P. orphoides*. Therefore, the members of the third subcluster can be specified as *Systemus* group.

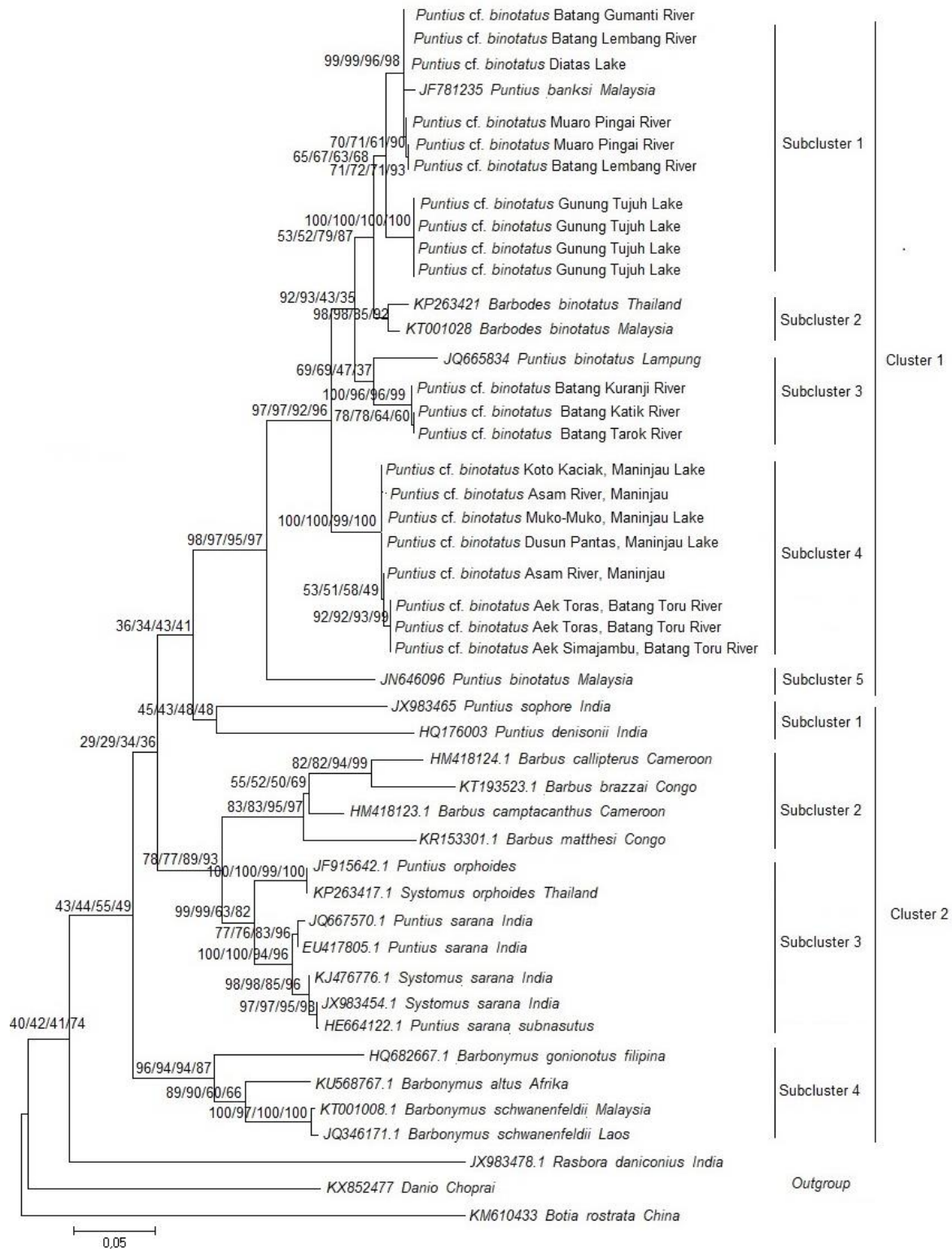


Figure 1. The ML phylogenetic tree of COI gene with bootstrapping of 1000 replicates (ME/ML/NJ/MP)

It is concluded that *P. cf. binotatus* group in Sumatra can be considered as a *Barbodes banksi* that further consisted of four subspecies, one of them is *P. cf. binotatus* Gunung Tujuh Lake named as *Barbodes banksi gunungtujuh*. In the present phylogenetic tree, there is a clear separation or grouping of each different genus in the

first cluster (*Barbodes*) and second cluster (*Barbus*, *Puntius*, *Systemus*, and *Barbonymus*). Moreover, the results of the analysis do not support the placement of *Barbodes*, *Barbus*, *Systemus*, and *Barbonymus* as synonyms of the *Puntius*.

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