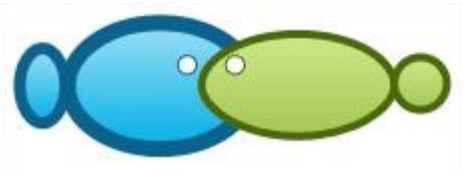

Scientific name confirmation of freshwater pufferfish (buntal) in Singkarak Lake, West Sumatra, Indonesia, based on cytochrome b and cytochrome oxidase subunit I gene

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Abstract. The pufferfish is one of the freshwater fish from the Tetraodontidae family, found in Singkarak Lake, West Sumatra, Indonesia, with the local name buntal or jabuih. Some scientific names have been used. Morphological identification is difficult to do because of the limited morphological characters. Besides, morphological characterization of specimens that have been fixed in formalin and preserved in ethanol is difficult to carry out. Therefore, identification is carried out through approaches using molecular characters. The study aimed to determine the proper name of the pufferfish from Singkarak Lake through the phylogenetic analysis based on the cytochrome b (Cyt b) gene and cytochrome oxidase subunit 1 (COI) gene. DNA sequences were aligned using Clustal X.2; the sequence divergence was analyzed using the Kimura 2-parameter (K2P) method, and the phylogenetic tree was analyzed using MEGA 7. The study results showed that the value of the genetic homogeneity level of pufferfish in Singkarak Lake was high (more than 90%). Based on the analysis of the segment sequence of Cyt b and COI genes (765 bp and 626 bp subsequently), it is known that pufferfish in Singkarak Lake, genetically is closely related to *Tetraodon cambodgiensis* and *Monotrete leiurus*. The phylogenetic tree constructed using these two genes showed that *Monotrete leiurus* has a shorter clade than *Tetraodon cambodgiensis*. In other words, *Monotrete leiurus* is a species that evolved earlier than *Tetraodon cambodgiensis*. Formerly, the name of *Monotrete leiurus* is *Tetraodon leiurus*. Therefore, the appropriate scientific name for the freshwater pufferfish in Singkarak Lake is *Tetraodon leiurus*, Bleeker 1850.
Key Words: COI, Cyt b, molecular identification, *Tetraodon leiurus*.

Introduction. Pufferfish is a type of fish that belongs to the Tetraodontidae family, distributed in tropical and subtropical regions. Pufferfish were commonly found in marine environments, but some species lived in brackish water and freshwater (Matsuura 2001; Nelson 2006; Froese & Pauly 2017). Dekkers (1975) reported the freshwater pufferfish were found in Singkarak Lake, West Sumatra. The local name of this species is buntal or jabuih. The scientific name of this species is still confusing, and the following names have been used: *Tetraodon leiurus* Bleeker, 1850 (Dekkers 1975), *Monotetra leiurus* Bleeker, 1850 (Roesma 2011), and *Tetraodon* sp. (Lubis et al 2012). The uncertainty of the scientific name caused the conservation status of this species also to be unclear.

Pufferfishes have a few external characters that are useful for taxonomy classification. The specimens are also easily distorted after being fixated in formalin and preserved in ethanol. Sometimes it is difficult for ichthyologists to recognize species and classify them into natural groups (Matsuura 2015). One way to solve the problem in the identification process is by using the molecular method. The mitochondrial DNA (mtDNA) genes are widely used as a molecular marker because of faster evolutionary rates than nuclear genes and are maternally inherited (no maternal and paternal recombination gene) (Arab et al 2017).

In vertebrate animals, the mtDNA size is around 16-20 kb and contains 37 genes (Khan et al 2017). Out of the mtDNA genes, the cytochrome b (Cyt b) and cytochrome

oxidase I (COI) genes are usually used for molecular analyses. For example, using both of the genes (Cyt b and COI), the population of silver barb from Gunung Tujuh Lake in Sumatra, which was previously classified as a member of the *Puntius*, has been confirmed as a *Barbodes banksi gunungtujuh* (Karlina et al 2016; Roesma et al 2019). Cytochrome b is a conservative protein-coding gene that shows more nucleotide variation in codon than some other genes in mtDNA (Arif & Khan 2009; Satoh et al 2016). The cytochrome b gene has been considered one of the most useful genes for phylogenetic study (Esposti et al 1993; Khan et al 2017). Phylogenetic analysis is very useful for distinguishing the taxa that are difficult to observe based on their morphological characters and can produce large amounts of data (Shearer & Coffroth 2008; Zimmer & Wildman 2017). Therefore the phylogenetic study has been widely used in settling various taxonomic problems (Farias et al 2001; Tang et al 2005; Igarashi et al 2013). One of the phylogenetic analyses carried out on pufferfish is on *Lagocephalus sceleratus* Gmelin, 1789. *L. sceleratus* is a pufferfish that migrated from the Mediterranean Sea and the Red Sea. The study results concluded that populations of both habitats do not show any genetic variation that will cause the speciation after migrating to new habitats (Farrag et al 2015).

The COI is another gene with the most conserved region in the mitochondrial genome and has a relatively short size, around 650 bp (Ward et al 2005; Magadam & Yousefi 2013). The COI gene sequence has been standardized and suitable to use as a DNA barcode at the various animal group's species levels (Kress et al 2015; Imtiaz et al 2017). DNA barcoding is a method to determining species based on the short gene sequences (Lebonah et al 2014; Kress et al 2015), which apply to identify organisms up to species level (Waugh 2007), to solve problem on cryptic species (Trivedi et al 2016), and to confirm the species identity that are morphologically similar (Ko et al 2013). The International Barcode of Life (iBOL) Project proposes using the DNA barcoding method as a global standard in identifying eukaryotic organisms (Jinbo et al 2011). The present study aimed to specify the scientific name of pufferfish in Singkarak Lake based on phylogenetic construction using the cytochrome b gene and DNA barcoding using the COI gene.

Material and Method

Sample sources and DNA extraction. Samples were obtained from three locations in Singkarak Lake (Muara Sumani as an inlet of Singkarak Lake, Dermaga Sumani as an area along the edge of the Singkarak Lake, and Ombilin River as an outlet of Singkarak Lake) in March 2018 (Figure 1). Individual samples were collected by direct survey and collection methods (Cailliet et al 1986). Liver tissue of pufferfish was collected and stored in 1.5 ml microtubes that contain ethanol PA (96%). The samples were preserved in 10% formalin for one week, then transferred to 70% ethanol for long-term preservation. The DNA sample was extracted from liver tissue according to Protocol Kit INVITROGEN PureLinkTM Genomic DNA Mini Kit. The DNA product was put to electrophoresis in 1.2% agarose at 100 V, 20 W for 30 min, and visualized under U.V. light.

Polymerase Chain Reaction (PCR) and DNA sequencing. The DNA product was amplified using a reaction mixture containing 25 µl total volume, 11 µl of 2x MyTaq Hs Red Mix (Bioline), 10 µl of ddH2O, 0.5 µl of each primer (0.01 mM), and three µl DNA template. The DNA was amplified using the following primer pairs; Cytball F1 (GACCTGT GGCGTGAAAAACC) forward primer and Cytball R1 (GTTTACAAGACCGGCGCTCT) reverse primer for the Cyt b gene (Igarashi et al 2013), and Fish F1 (TCAACCAACCACAAAGACA TTGGCAC) forward primer and Fish R2 (ACTTCAGGGTGACCGAAGAATCAGAA) reverse primer for the COI gene (Ward et al 2005). The PCR process for the Cyt b gene consisted of: pre denaturation (94°C for 3 min), denaturation (94°C for 0.5 min), annealing (55°C for 2 min), elongation (72°C for 1 min), and final extension step (72°C for 5 min) (Igarashi et al 2013). The PCR process for the COI gene consisted of: predenaturation (95°C for 2 min), denaturation (94°C for 0.5 min), annealing (55°C for 0.5 min),

elongation (72°C for 1 min), and the final extension step (72°C for 10 min) (Roesma et al 2018).

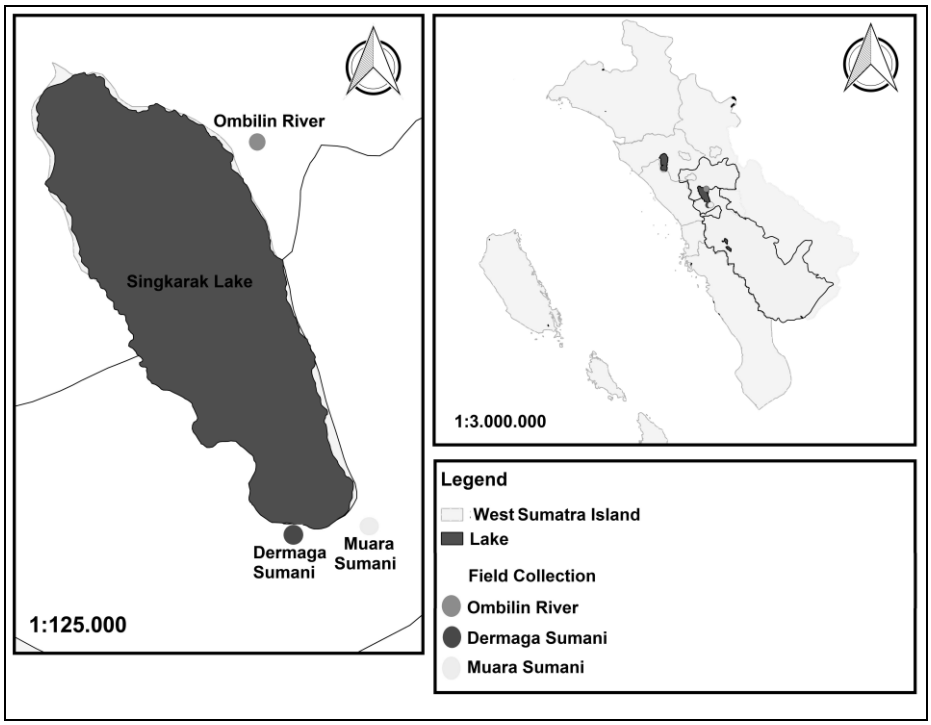

Figure 1. Sampling areas in Singkarak Lake (map generated using QGIS v 3.24 software).

All PCR products are held at 4°C for long-term storage. The PCR product was visualized using electrophoresis on 2% agarose gel and purified before sequencing to the MacroGen DNA, South Korea.

Data analysis. DNA sequences forward and reverse of pufferfish in Singkarak Lake were contig using the DNA STAR program (Burland 2000) and compared with observing the similarity of DNA sequences in GenBank of National Center of Biotechnology Information (NCBI 2018) (Table 1). A total of 24 accessions of Cyt b and COI from GenBank, respectively (Tetraodontidae, Diodontidae, Molidae, Ostraciidae, and Cyprinidae (outgroup)) were downloaded as data comparison (Table 1). DNA sequences of pufferfish were arrenged using Clustal X software (Thompson et al 1997). The Bioedit program was used to edit and check the sequences after alignment (Hall 1999). The number of polymorphic sites and haplotype diversity was determined using DNA Sequence Polymorphism 5.10 (Rozas et al 2003). The phylogenetic tree was arranged using the MEGA 7 (Molecular Evolutionary Genetics Analysis) software program (Kumar et al 2016).

Table 1

Information list of species, location, sources, and GenBank accession number in the present study

Species	*Sample Information*		*Sources*		*GenBank Accession no.*	
	Origin of specimen Cyt b	*Origin of specimen COI*	*Cyt b*	*COI*	*Cyt b*	*COI*
Tetraodon nigroviridis	-	South America	-	(Amaral et al 2013)	-	KC959930.1
Tetraodon nigroviridis	Thailand	Southeast Asia	(Sangthong et al 2013) Unpublished	(Santini et al 2013)	KF781161.1	JQ681838.1
Tetraodon fluviatilis	-	Southeast Asia	-	(Santini et al 2013)	-	JQ681833.1

Tetraodon biocellatus	-		-	(Khedkar et al 2012) Unpublished	-	JQ667577.1
Tetraodon biocellatus	-	South America	-	(Amaral et al 2013)	-	KC959929.1
Tetraodon baileyi	Asia	-	(Igarashi et al 2013)	-	AB741978.1	-
Tetraodon abei	Asia	-	(Igarashi et al 2013)	-	AB741977.1	-
Tetraodon palembangensis	Southeast Asia	Southeast Asia	(Santini et al 2013)	(Santini et al 2013)	JQ681932.1	JQ681839.1
Tetraodon erythrotaenia	-	Southeast Asia		(Santini et al 2013)		JQ681832.1
Tetraodon turgidus	Asia	-	(Igarashi et al 2013)	-	AB741992.1	-
Tetraodon suvattii	Asia	-	(Igarashi et al 2013)	-	AB741991.1	-
Tetraodon lineatus	Southeast Asia	-	(Santini et al 2013)	-	JQ681928.1	-
Tetraodon lineatus	Asia	-	(Igarashi et al 2013)	-	AB741986.1	-
Monotrete leiurus	Asia	Southeast Asia	(Igarashi et al 2013)	(Santini et al 2013)	AB741985.1	JQ681834.1
Monotrete cochinchinensis	Asia	Southeast Asia	(Igarashi et al 2013)	(Santini et al 2013)	AB741980.1	JQ681829.1
Tetraodon cambodgiensis	Southeast Asia	Cambodia	(Santini et al 2013)	(Santini et al 2013)	JQ681921.1	JQ681828.1
Arothron hispidus	Southeast Asia		(Santini et al 2013)	(Santini et al 2013)	JQ681857.1	JQ681763.1
Arothron hispidus	Taiwan	Reunion, Madagascar, France, and Polynesia	(Hsieh & Chung 2008) Unpublished	(Hubert et al 2012)	FJ434546.1	JQ431462.1
Arothron nigropunctatus	-		-	(Santini et al 2013)	-	JQ681767.1
Arothron nigropunctatus	-	Canada	-	(Steinke et al 2009)	-	FJ582880.1
Arothron nigropunctatus	-	Lizard Island, Australia	-	(Steinke et al 2014) Unpublished	-	KP194217.1
Arothron mappa	Southeast Asia	Reunion, Madagascar, France,Polynesia	(Santini et al 2013)	(Hubert et al 2012)	JQ681860.1	JQ349779.1
Lagocephalus lunaris	Thailand	-	(Sangthong et al 2013) Unpublished	-	KF781150.1	-
Lagocephalus lunaris	Thailand	-	(Sangthong et al 2013) Unpublished	-	KF781151.1	-
Takifugu rubripes	Japan	-	(Yanagimoto et al 2016) Unpublished	-	LC205862.1	-
Takifugu rubripes	Japan	-	(Yanagimoto et al 2016) Unpublished	-	LC205854.1	-
Canthigaster compressa	-	Lizard Island, Australia	-	(Steinke et al 2014) Unpublished	-	KP194162.1
Canthigaster compressa	-		-	(Santini et al 2013)	-	JQ681774.1
Diodon holocanthus	Japan	-	(Santini et al 2013)	(Bernard et al 2017) Unpublished	JQ681853.1	MF041658.1

Diodon holocanthus	Japan	-	(Yamamoue et al 2006)	(Bernard et al 2017) Unpublished	AP009177.1	MF041608.1
Mola mola	Taiwan	-	(Jean 2006) Unpublished	-	AM265578.1	-
Mola mola		-	(Santini et al 2013)	-	KF025731.1	-
Tetrosomus concatenatus	-		-	(Steinke et al 2011) Unpublished	-	JF494670.1
Tetrosomus concatenatus	-		-	(Steinke et al 2011) Unpublished	-	JF494669.1
Cyprinus carpio	India	Canada	(Dwivedi et al 2013) Unpublished	(Schoelinck et al 2016) Unpublished	KF574490.1	KX145549.1
Danio dangila	-	Bangladesh	-	(Kullander et al 2015)	-	KT199753.1
Rasbora daniconius	India	-	(Singh et al 2013) Unpublished	-	KF574538.1	-

Results

Phylogenetic analysis and species identity. The total number of 24 sequences was obtained from the GenBank as the comparison data in the phylogenetic analysis. Analysis carried out using the COI and Cyt b sequences from pufferfish in Singkarak lake and the Cyt b and COI sequences for pufferfish that are available in the GenBank, NCBI. Five pufferfish in Singkarak Lake were used for the Cyt b gene and six individuals for the COI gene analysis. The characteristics of Cyt b and COI gene sequences of the pufferfish in Singkarak Lake are shown in Table 2. The results were similar to previous studies, where base compositions in fish are A (adenine) 24.8%, T (thymine) 29.5%, C (cytosine) 30.5%, and G (guanine) 15.3% (Satoh et al 2016). The number of A+T bases higher than G+C bases, was also found in *Tetraodon lineatus* (Gong et al 2016) and *Tetraodon miurus* (Yamanoue et al 2011). The total number of A+T in the vertebrate group is higher than G+C because the rate of mutations (transitions and transversions) in mtDNA is relatively high (Belle et al 2005).

Table 2

Molecular data characteristics of pufferfish in Singkarak Lake

No.	*Molecular analysis*	*Cyt b*	*COI*
1	The total length of the sequences	1183-1311 bp	776-864 bp
2	The total length of the analyzed	765 bp	626 bp
3	Conserved site	428 bp	403 bp
4	Variable site	337 bp	223 bp
5	Parsimony site	302 bp	207 bp
6	Singleton site	35 bp	16 bp
7	Adenine base composition	24.6%	24.3%
8	Thymine base composition	26.2%	26.2%
9	Cytosine base composition	34.3%	30.7%
10	Guanine base composition	15.0%	18.8%
11	Adenine + Thymine	50.5%	50.5%
12	Guanine + Cytosine	49.5%	49.5%
13	Total of Haplotype	26	23
14	The genetic distance of intraspecific	0.0%-0.1%	0.0%

Sequence similarity analysis using BLAST showed that the pufferfish sequences in Singkarak Lake occupy the accurate position on the mtDNA of *M. leiurus*. The Cyt b gene sequence of pufferfish in Singkarak Lake (765 bp) was obtained in position 14431-15196

bp from the complete mtDNA genome of *M. leiurus*. The sequence range is the Cyt b region of *M. leiurus*. The present study result is in line with Hu et al (2015), which state that the *M. leiurus* cytochrome b gene is at position 14346-15482 bp of the complete mitochondrial DNA genome.

The COI gene sequence from the six individuals pufferfish in Singkarak Lake obtained ranged from 76-864 bp. The 626 bp out of them was used for analysis. The position of the bases lies between 5523-6148 bp of the *M. leiurus* mitochondrial complete genome. According to Hu et al (2015), the position of the COI gene in the mitochondrial genome of species *M. leiurus* is between 5451-7010 bp. The sequences analyzed of pufferfish Singkarak Lake are at a position 72-697 bp of the COI gene in *M. leiurus* species.

Based on the analysis using DNA Sequence Polymorphism obtained, there are 26 haplotypes from 29 sequences of the Cyt b gene. A haplotype is a combination of alleles at different markers along the same chromosome inherited as a unit (Crawford & Nickerson 2005; Templeton 2005). Out of 26 haplotypes, pufferfish in Singkarak Lake consists of two haplotypes, Haplotype 7 and Haplotype 8. Pufferfish from Dermaga Sumani and Muara Sumani shared the same haplotype (Haplotype 7). Meanwhile, pufferfish from the Ombilin River population shared the Haplotype 8. The difference between Haplotype 7 and Haplotype 8 is present on the 413th base, whereas the base has changed from the Cytosine to Thymine. The sequence divergence between five individuals ranges from 0.0%-0.1%. Referring to Kartavtsev (2011), the difference between Haplotype 7 and Haplotype 8 indicates that they are at the level of variation in the same species.

The analysis using the COI gene shows that there are 23 haplotypes out of 30 sequences. All pufferfish populations in Singkarak Lake shared the same haplotype (Haplotype 15) with 0.0% sequence divergences. According to Kartavtsev (2011), the difference sequences divergence value of animals in the COI gene at the species level in the population is 0.89±0.16%. Therefore, based on haplotype information (Cyt b and COI genes), we can conclude from the present study that pufferfish from Singkarak Lake have a high genetic homogeneity level (more than 90%).

The phylogenetic tree of the pufferfish in Singkarak Lake was constructed with four methods; Maximum Likelihood (ML), Neighbour Joining (NJ), Minimum Evolution (ME), and Maximum Parsimony (MP) using MEGA 7, which is represented by the ML tree. The sequence divergence value of pufferfish in Singkarak Lake and the closed relative is not shown. Figure 2 showed that the phylogenetic tree was divided into two main clusters, supported by bootstrap 67/74/73/57(ML/NJ/ME/MP). Bootstrap values at each branching point of the phylogenetic tree explain the level of species separation. The first cluster consists of Tetraodontidae, and the second cluster consists of Diodontidae and Molidae. The first and second clusters belong to Tetraodontiformes and are grouped monophyletically against its outgroup (Cyprinidae family Cypriniformes).

The first cluster is divided into five sub-clusters, supported by bootstrap value 66/73/70/57 (ML/NJ/ME/MP). In the first sub-cluster, pufferfish from Singkarak lake grouped monophyletically with *Monotrete* and *Tetraodon*, supported by bootstrap 98/89/99/99 (ML/NJ/ME/MP). The member of this sub-cluster is the pufferfish that lives in freshwater (Baran et al 2013; Doi et al 2015; Saenjundaeng et al 2015). The value of the sequence divergences in this sub-cluster was 0.1%-4.2%. Based on Kartavtsev (2011; 2013), the value of sequences divergence placed the pufferfish in Singkarak Lake at the same species-level as *Tetraodon* and *Monotrete*. Therefore, all the species in the first cluster were sister taxa. Sister taxa are closed relatives of species from the same branch of the phylogenetic tree.

The value of sequence divergences between pufferfish from Singkarak Lake with *T. Cambodgiensis* is 0.9%-1.1% and 1.1%-1.2% with *M. Leiurus* in the first sub-clusters,

with a bootstrap value of 99/99/98/95 (ML/NJ/ME/MP). Based on the range of sequence divergence values, it can be considered that the pufferfish from Singkarak Lake is at the same species level as *T. cambodgiensis* and *M. leiurus*. Based on Kottelat (2013), *M. leiurus* in this sub-cluster was placed into a new genus as *Pao*, indicates that the first sub-cluster contains pufferfish from the genus *Pao*.

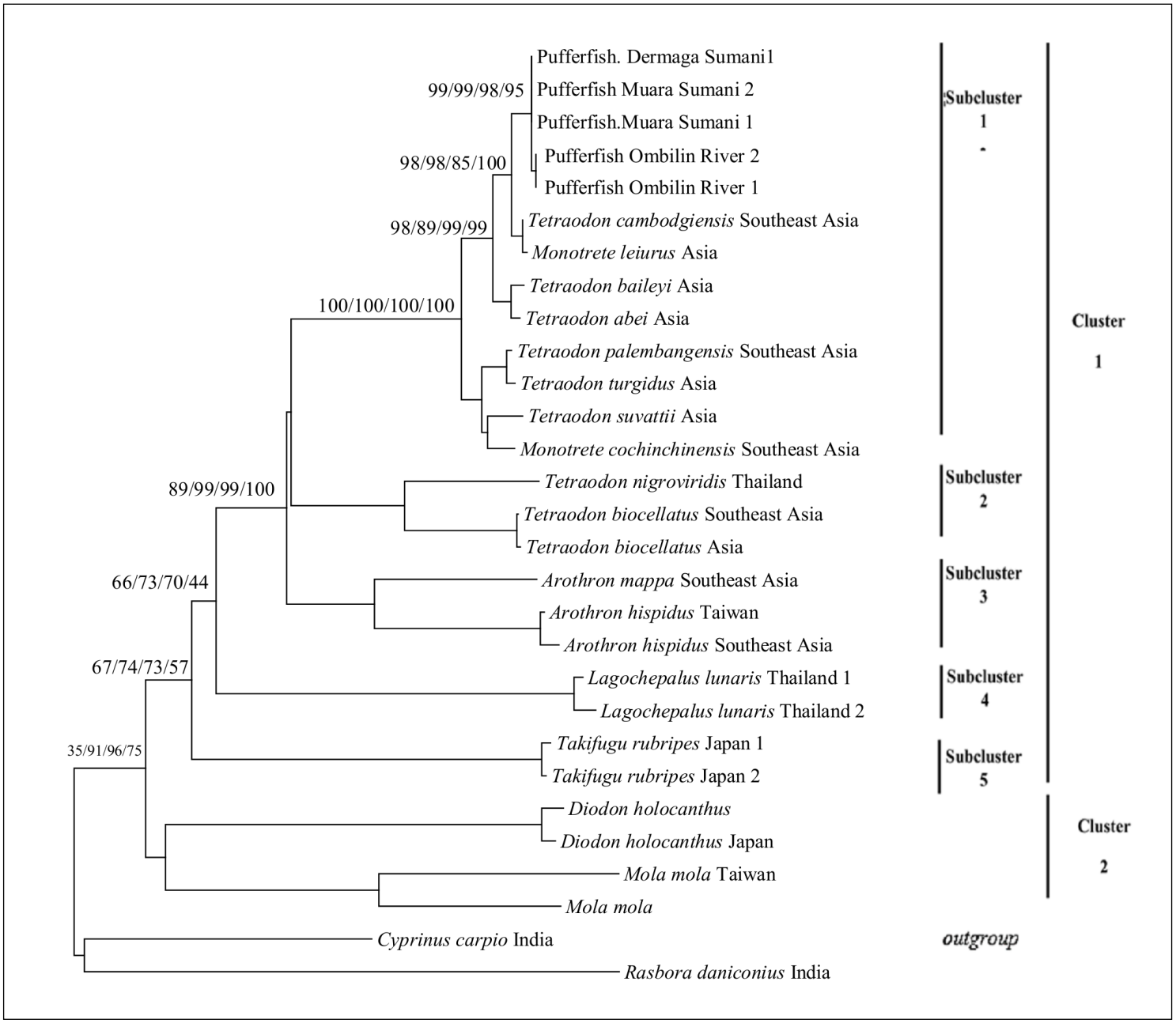

Figure 2. The phylogenetic tree (ML) of pufferfish in Singkarak Lake and the close relatives based on the cytochrome b gene with 1000 bootstrapping (ML/NJ/ME/MP).

The second sub-cluster consists of *T. biocellatus* and *T. nigroviridis*. Both are the type of pufferfish that can live in fresh and brackish water (Yamanoue et al 2011; Shamsuzzaman et al 2015). They have a sequence divergence of 13.7%-14.9% - with pufferfish from Singkarak Lake. Referring to Kartavtsev (2011; 2013), that value put the pufferfish in Singkarak Lake at the same genus level as the pufferfish in the second sub-cluster. Kottelat (2013) proposed two new genera, *Pao* for *Tetraodon* and *Monotrete*, and Dichotomyctere for *Tetraodon biocellatus* and *Tetraodon nigroviridis*. However, referring to Kartavtsev (2011; 2013), the sequence divergence in the two subclusters (0.1%-14.9%) shows that they are not in a different genus.

The members of the third sub-cluster, consist of *Arothron mappa* and *A. hispidus*. The two species were found in brackish water and seawater (Matsuura 2001). Based on the phylogenetic tree, pufferfish in Singkarak Lake were not at the same level as *Arothron* because of the sequence divergences ranging from 14.5%-16.7%. This value is close to the sequence divergence at the different genera in the same family of 17.86±1.36% (Kartavtsev 2011). Morphologically, *Arothron* and *Tetraodon* are different in nasal organs. *Arothron* had a nasal organ with two nostrils and two thick tentacles. It was not covered by the small sac. Meanwhile, the nasal organ in a *Tetraodon* species forms like a tube with a single nostril at its end (Weber et al 1962; Matsuura 2001).

Pufferfish in Singkarak Lake showed a paraphyletic relationship to pufferfish in the fourth and fifth sub-cluster. Pufferfish in the fourth and fifth sub-clusters are the type that lives in the sea (Shamsuzzaman et al 2015). The sequence divergences between pufferfish in Singkarak Lake and them was 20.3%-20.7%. Based on Kartavtsev (2011; 2013), the value indicates that pufferfish in Singkarak Lake, pufferfish in the fourth and the fifth sub-cluster are different at the same family level (Tetraodontidae).

The phylogenetic tree (Figure 2) also showed that pufferfish clustered according to their habitat. Freshwater pufferfish clustered in the first sub-cluster, pufferfish that can live well in freshwater or estuary clustered in the second sub-cluster, and sea pufferfish clustered in the third, fourth, and fifth sub-cluster. According to Igarashi et al (2013), the pufferfish is divided into the separated group consisting of: the Asian freshwater group (Matsuura 2001), the Asian brackishwater group (Nelson 2006), and the African freshwater group (Froese & Pauly 2017).

The phylogenetic tree in Figure 3 was constructed using cytochrome oxidase 1 (COI) gene sequences. The sequence divergence value of pufferfish in Singkarak Lake and the closed relatives is not shown. Figure 3 showed the phylogenetic tree is divided into two main clusters consisting of two sub-clusters, respectively. The first sub-cluster in the first cluster consists of pufferfish in Singkarak Lake, *T. cambodgiensis*, *M. leiurus*, *M. cochinchinensis*, and *T. palembangensis*. The sequence divergence of pufferfish in Singkarak Lake to *T. cambodgiensis* and *M. leiurus* were 0.32% and 0.48%. According to Ward et al (2005) and Kartavtsev (et al 2009; 2011; 2013), the value showed that pufferfish in Singkarak Lake and those two species are at the level of genetic variation within species.

The pufferfish in Singkarak Lake has a monophyletic relationship with *M. cochinchinensis*, and *T. palembangensis*, with sequence divergence values of 3.67%, and 3.83%. According to Ward et al (2005) and Kartavtsev (et al 2009; 2011; 2013), the pufferfish in Singkarak Lake should be as sister taxa (subspecies) of *M. cochinchinensis* and *T. palembangensis*.

Arothron hispidus, *A. mappa*, and *A. nigropunctatus* in the second sub-cluster in the first cluster are marine pufferfish. The sequence divergences of the pufferfish in Singkarak Lake and those species are 13.10%-14.70%. Based on Kartavtsev (2011), the sequence divergences (COI gene) between different species from the same genus is 11.06±0.53%. Therefore, pufferfish in Singkarak Lake are species in the same genus with different morphology to pufferfish in the second sub-cluster. *Tetraodon* has a black spot (Weber et al 1962), while *Arothron* has a white spot (Randall et al 2011).

Canthigaster compressa in the first sub-cluster of the second cluster is also a pufferfish from the sea. The sequence divergences between pufferfish in Singkarak Lake and *C. compressa* is 13.58%. According to Ward et al (2005) and Kartavtsev (et al 2009; 2011; 2013), the value supported that the pufferfish from Singkarak Lake and *C. compressa* are species in the same family (Tetraodontidae). *T. biocellatus*, *T. erythrotaenia*, *T. nigroviridis*, and *T. fluviatilis* in the second sub-cluster of the second cluster are brackish water pufferfish. The sequence divergence between pufferfish in Singkarak Lake and those species is 14.06%-15.02%. Ward et al (2005) and Kartavtsev (et al 2009; 2011; 2013) concluded that pufferfish in Singkarak Lake are species in the same genera as those groups.

Diodon holocanthus (Diodontidae) and *Tetrosomus concatenatus* (Ostraciidae) from Tetraodontiformes also was included in the COI tree. Both of them are marine pufferfish. The sequence divergence between pufferfish in Singkarak Lake with *D.*

holochantus and *T. concatenatus* is 19.49% and 18.21%-18.69%. According to Ward et al (2005) and Kartavtsev et al (2009; 2011; 2013), the result of the analysis is congruence with the branching in the tree.

The sequence divergence value will increase as species taxon levels rise (Kartavtsev et al 2009; 2011; 2013). The sequence divergence values can determine the

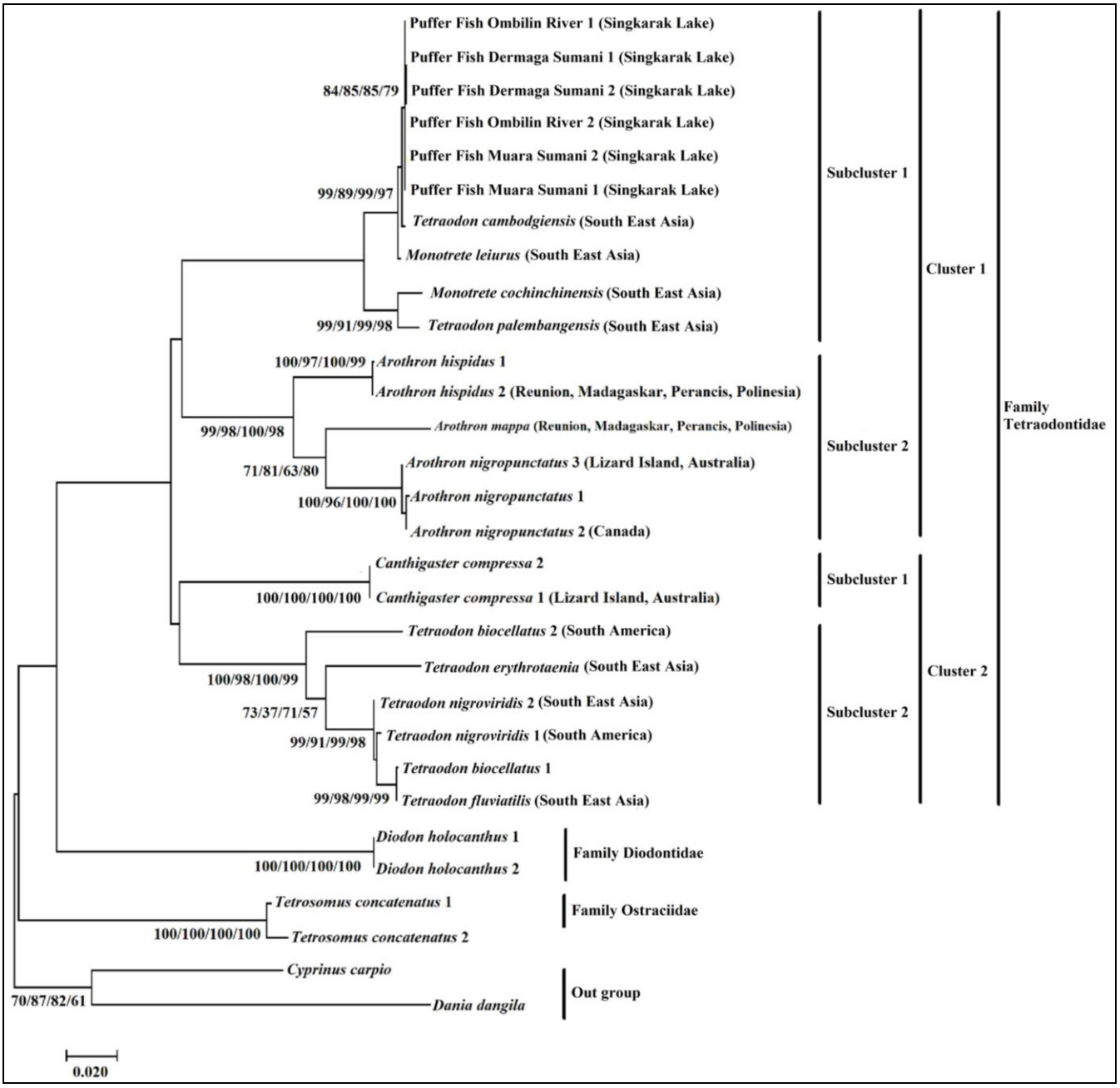

Figure 3. The phylogenetic tree (ML) of pufferfish from Singkarak Lake and the closed relative based on the COI gene with 1000 bootstrapping (ML/NJ/ME/MP).

position of a species in the taxonomy. The lower the value of sequence divergence, the closer the relationship of species, and vice versa. Following the values inferred by Kartatvsev (et al 2009; 2011; 2013), sequence divergences value will increase with the ascent of species level. The same species of Tetraodontidae have low sequence divergences meanwhile, the different species have high sequence divergences. It can be seen at intraspecific value, i.e. pufferfish in Singkarak Lake (0.0%), *A. hispidus* (0.16%), *A. nigropunctatus* (0.16%-0.32%), *D. nigroviridis* (0.32%), and *C. compressa* (0.0%).

The result reinforces the statement that the COI gene is suitable for species identification.

Scientific name analysis. Individual samples of pufferfish were collected at three points on Singkarak Lake; Muara Sumani (an inlet of Singkarak Lake), Dermaga Sumani (the edge of Singkarak Lake), and Ombilin River (outlet of Singkarak Lake). Based on Cyt b gene analysis, genetic distance from the five samples of pufferfish in Singkarak Lake ranged from 0.0%-0.1%. Based on COI gene analysis, the pufferfish in Singkarak Lake has a genetic distance of 0.0%. The value shows that pufferfish in Singkarak Lake is the population with a more than 90% homogeneity value.

Based on the phylogenetic tree (Figure 3) using the COI gene, *M. leiurus* has a shorter clade than *T. cambodgiensis*. According to Gregory (2008), the clade in the phylogenetic tree could interpret the evolution time of species. The shorter a clade, the earlier a species evolved. It means that *M. leiurus* is a species that has evolved earlier than *T. cambodgiensis*.

Morphologically, *T. cambodgiensis* has a different color pattern from *M. leiurus* (Saenjundaeng et al 2013). *T. cambodgiensis* has a pattern of one large black spot surrounded by white color (white-spot cambodgiensis-pattern) or ocellus near the caudal fin. Black spots on this type of fish are on the right and left sides of the body and the dorsal (Dekkers 1975). Black spots in *M. leiurus* are found on the right and left sides of the body up to the abdomen and do not have a large ocellus (Rainboth 1996). *T. cambodgiensis* is distributed in Thailand, Cambodia, and Vietnam precisely on the Mekong River (Kartavtsev 2011; Kottelat 2011) and not in Indonesia (Vidthayanon 2012). *M. leiurus* is a native species of Indonesia, found in Singapore, Sumatra (Singkarak Lake, rivers in Solok, and Palembang), Borneo (Kapuas), and Java (rivers in Bogor and Jakarta) (Weber et al 1962; Dekkers 1975; Froese & Pauly 2017). Morphologically, pufferfish in Singkarak Lake have the black spot pattern as *M. leiurus*, where these fish do not have ocelli. Their black spot pattern is found on the body side of the abdomen. Based on the description, it can be stated that pufferfish in Singkarak Lake is a type of *M. leiurus*, however *M. leiurus* has undergone a scientific name change. The name that has been used for this type is *Tetraodon leiurus* Bleeker, 1850, *Tetrodon leiurus* Bleeker, 1850, *Monotetra leiurus* Bleeker, 1850, *Monotrete leiurus* Bleeker, 1850, and *Pao leiurus* Bleeker, 1850. Referring to Dekkers (1975), the name of *Tetraodon leiurus* Bleeker, 1850 belongs to pufferfish in Java (Syntype). The 'Tetraodon' word comes from the 'Tetra' (four pieces) and 'Odont' (teeth). While 'Leiurus' is derivate from the Greek 'Leios', meaning smooth. This word refers to specimens of pufferfish that have fine thorns on the surface of their bodies. Spines in this type of pufferfish are smaller than *T. fluviatilis* group specimens. The name *T. leiurus* means fish has four large teeth and fine spines on its body surface.

The name of *Tetraodon leiurus* Bleeker, 1850 subsequently changed to *Monotrete leiurus* Bleeker, 1850. The word *Monotrete* comes from Latin, which means 'Mono' (one) and 'Trete' (pierced) (Kottelat 2001). This word shows the characteristics of pufferfish, which have spots or points on the surface of the body near the caudal fin (Dekkers 1975; Matsuura 2001). Then Kottelat (2013) placed *Monotrete leiurus* into the new genus *Pao*. The word *Pao* comes from a combination of Pla Pao and Pa Pao (Thailand and Laos). The words 'Pla' and 'Pa' mean fish, while Pao means a bag, so the word Pao is interpreted as marsupial fish (Kottelat 2013). The meaning of the word Pao shows the morphological characteristics of pufferfish that are like sacks. If all pufferfish have to use the *Pao* name to show their morphological characteristics, then all Pufferfish members in the Tetraodontidae family are placed in the genus *Pao*. However, in the genetic distance, the sequence divergences value of Pufferfish between sub-clusters shows that these species are not at the same genus level. Based on this description, the proper scientific name for pufferfish in Singkarak Lake (Weber et al 1962; Dekkers 1975) is *Tetraodon leiurus* Bleeker, 1850.

DNA barcode. The sequence of the COI gene was used to design the DNA barcode of pufferfish in Singkarak Lake. Pufferfish populations in Singkarak Lake have a different

DNA barcode pattern with other freshwater pufferfish on some bases, which are presented in Table 3.

Pufferfish in Singkarak Lake were at the same species level as *T. cambodgiensis* and *M. leiurus,* which differ in a nucleotide base (390th). Pufferfish in Singkarak Lake have nucleotide base adenine (codon: GGA) on the 390th base, while *T. cambodgiensis* and *M. leiurus* have nucleotide base guanine (codon: GGG). Pufferfish in Singkarak Lake, is the sister taxa of *M. cochinchinensis* and *T. Palembangensis* and has more differences in nucleotide bases (Table 3). Thus, the DNA barcode of pufferfish from Singkarak Lake is shown in Figure 4.

In the present study, of all sequences difference, we know that the transition is bigger than transversion. Transition is the interchanges of purine to purine or pyrimidine to pyrimidine (Luo et al 2016). A transversion is the interchanges of purine to a pyrimidine or pyrimidine to purine. Based on the reference (Smith & Simmonds 1997), purine and pyrimidine have different conformations. Purine has a bicyclic structure, and pyrimidine has a single ring structure. The structural differences were caused by the transversion process, more complicated than the transition process, so the opportunity of a transversion is lower than the transition. All nucleotide bases in the DNA barcode were translated to an amino acid. Based on the amino acid translation, we know that all the codons of the DNA barcode of pufferfish in Singkarak Lake and other species have the same amino acids. It indicated that the silent mutation occurred. A silent mutation is a differentiation of the nucleotide base, but the amino acids produced do not change (Obuya et al 2015).

The present study suggests that the COI gene is proven suitable for use as a DNA barcode because although there are variations in the sequence, it does not change the amino acid. Based on the description, we know that the DNA barcode that used the COI gene is specific and suitable in the molecular marker for species identification. Based on Turan et al (2017), the design of DNA barcoding from the COI gene data is provided for pufferfish identification. In line with Imtiaz et al (2017) who also stated that DNA barcoding has a high potential to identify species into taxa and resolves the ambiguousness in species identification.

Table 3

Variables site of pufferfish in Singkarak Lake and other freshwater pufferfish in DNA barcode

Sequences base	*6 9*	*2 5 2*	*2 7 6*	*3 9 0*	*3 9 3*	*4 0 5*	*4 0 8*	*4 7 4*	*5 2 2*
Pufferfish in Singkarak lake	A	T	A	A	G	G	T	G	T
Tetraodon cambodgiensis	.	.	.	G	.	.	.	.	.
Monotrete leiurus	.	.	.	G	.	.	.	.	.
Monotrete cochinchinensis	T	C	.	G	C	A	C	A	C
Tetraodon palembangensis	C	C	G	G	C	A	C	A	.

Figure 4. DNA Barcode of pufferfish from Singkarak Lake, West Sumatra.

Morphology characters. Characters: rounded body, scaly body side, small spines, muzzle 2x shorter than the head length, nasal organs united at the base, two-pronged

high nostrils, flat head, lower edge of eye equal to mouth, the average total length 102.39 mm, the average of fork length 89.02 mm, the average of standard length 83.52 mm, the average of preorbital length 14.86 mm, the average of eye diameter 6.59 mm, the average of head length 29.48 mm, the average of minimum body width 26.92 mm, 10-13 dorsal-fin rays, 20 pectoral-fin rays, 1 spine and 9-11 rays of the anal fin, black dots at dorsal and white at ventral.

Local distribution: Ombilin River, Singkarak Lake at Sumpur, Sumani, Paninggahan, Muaro Pingai (Solok). Geographic distribution: Sundaland, Indochina (Kottelat et al 1993).

Conclusions. The pufferfish in Singkarak Lake collected from the three locations, had a high genetic homogeneity level of more than 90%. Based on the phylogenetic analysis using cytochrome b and COI genes and their description, the proper scientific name for pufferfish in Singkarak Lake is *Tetraodon leiurus* Bleeker, 1850. DNA barcoding supports the result of phylogenetic analysis.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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