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Molecular characterization of Tapah fish (*Wallago leerii*, Bleeker 1851) from Riau Province based on Cytochrome b gen

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ABSTRACT

Tapah fish (*Wallago leerii*) is a fish with high economic value in Riau Province. Molecular research on *W. leerii* is very important to do. This study aims to determine the molecular characteristics of *W. leerii* from Riau Province based on the Cytochrome b gene. The universal primer Cytochrome b is used for the Polymerase Chain Reaction (PCR) process. The PCR result is a partial Cytochrome b gene with a length of 373 bp. Multiple alignments were performed on nucleotide sequences of the Cytochrome b gene of *W. leerii* from river of Tapung and Indragiri Riau with the cytochrome b gene of other fish from Genbank and analyzed using the MEGA program version 6.0. Phylogenetic construction using the Cytochrome b gene can distinguish *W. leerii* from river of Tapung and Indragiri Riau, with other species.

Introduction

Riau Province is a geographical area that has four major rivers. These rivers include the Kampar, Siak, Indragiri and Rokan Rivers. The river in Riau Province has the characteristics of a flood plain river (Elvyra, 2009).

The flood plain river ecosystem is a complex system consisting of riverbeds, tributaries, and flooded lakes (Rollet *et al.*, 2014). This complex system has certain functions to fulfill the liberation of fish life in that habitat. The bottom of the river is used as a shelter for fish from predators; tributaries, especially on the banks of the river, can be used as shelter and foraging; flooded lakes are used by fish as a place of refuge, foraging, spawning and laying eggs until they hatch because there is riparian vegetation which is submerged when the rainy season enters (Hartoto *et al.*, 1998).

The Tapah fish (*Wallago leerii* Bleeker, 1851) is a unique fish that inhabits flood plain river areas, including the Kampar, Tapung and Indragiri Rivers in Riau Province. According to Kottelat *et al.* (1993), *W. leerii* are included in the catfish group or fish that have antennae. In the classification structrure, the *W. leerii* belongs to the order Siluriformes, family Siluridae, genus Wallago and species *W. leerii*.

W. leerii is a fish that has high economic value and have a maximum weight of up to 35 kg and total length 1.5 m (Kottelat *et al.*, 1993). Catches that are continuously carried out by fishermen and no conservation efforts are carried out, it is feared that extinction will occur in the future.

The development of molecular techniques is currently very helpful in obtaining scientific information genetically. Genetic information obtained can be used to differentiate species and see kinship and diversity between species (Muchlisin *et al.*, 2017; Yulianto *et al.*, 2020; Batubara *et al.*, 2021). Polymerase Chain Reaction (PCR) is a method that can be used to reproduce the DNA of an organism (Garibyan and Avashia, 2013). PCR-based methods are often used in the identification of organisms, either through DNA finger printing or through DNA barcoding (Hebert *et al.*, 2004). Genetic identification

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methods using the PCR method have been developed a lot, and have been carried out on various marine organisms, including various corals hard (Shearer and Coroth, 2008).

To reveal the genetic information of *W. leerii*, Cytochrome b (cyt-*b*) markers were used. Previous studies have used cyt-*b* extensively to reveal the divergence of fish species at several taxonomic levels, such as the Cichlidae family (Farias *et al*., 2001), the Actinopterygian group (Lydeard and Roe, 1997; Sevilla *et al*., 2007), the Goodeidae family. (Doadrio and Dominguez, 2004), order Gadiformes (Akasaki *et al*., 2006), family Melanotaeniidae (Zhu et a., 1994), family cyprinidae (He and Chen, 2007), genus Lethtrinus (Lo-Galbo *et al*., 2002), and family Salangidae (Zhang *et al*., 2007), while genetic studies of *W. leerii* from Riau Province, Indonesia using cyt-*b* markers have never been reported, so it is important to disclose.

Materials and Methods

Location and time of research

This research was conducted in December 2017 - May 2018 at the Genetics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Riau. Sampling was obtained from fishermen as many as 30 fish samples (10 individuals per species) from three different rivers, namely the Kampar (0°11'38" N, 101°28'59" E), Tapung (0°40'07" N, 101°17'16" E) and Indragiri (0°24'16" S, 102°36'45" E) Rivers of Riau Province.

mtDNA Extraction and Isolation

In the mtDNA extraction and isolation process, three fish samples were taken from 30 individuals in each river. The sample was taken in the form of fish muscle tissue near the tip of the tail which was cut with a size of 1 cm. The tissue is stored in absolute alcohol. The extraction and isolation processes refer to the Dneasy Blood and Tissue Kit (Qiagen) DNA isolation and purification kit protocol.

Fish muscle tissue was dried and finely chopped, added with TE Buffer, vortexed for 15 seconds, then centrifuged at 3000 rpm for 2 minutes. Discard the TE Buffer liquid and dry the sample and then put into a 1.5 ml tube. The sample was added with 180 µl of ATL buffer to lyse the tissue. After being lysed, the samples were vortexed with 20 µl Proteinase K for 10 seconds and incubated for 1-2 hours in a water bath at 56°C until all samples dissolved.

Samples that had been incubated were removed and vortexed 200 µl AL buffer for 15 seconds, then 200 µl absolute ethanol was added and vortexed for 2 minutes. After that, the sample was transferred to a 2 ml spincolumn, centrifuged at 8000 rpm for 1 minute. Next, discard the column tube which contains the supernatant.

In the washing process, add 500 µl of Buffer AW 1, centrifuge at 8000 rpm for 1 minute. Then added 500 µl of Buffer AW 2 and centrifuged at 13000 rpm for 3 minutes. After that, 200 µl of AE Buffer was added to obtain total DNA stock, incubated at room temperature for 1 minute and centrifuged at 8000 rpm. The DNA stock that has been obtained is stored at 4°C.

Electrophoresis DNA extraction product

Visualization of DNA bands can be done by electrophoresis method. The medium used was made using 1% agarose gel solution, 1X TBE buffer (Tris-borate-EDTA pH 8.0) and 1 µg/ml ethidium bromide heated until dissolved and poured into the mold. The electrophoresis process was carried out at a voltage of 50 volts for 30 minutes. Electrophoresis results can be observed using a UV transilluminator and photographed with a camera.

Cytochrome b gene amplification from mitochondrial DNA

The primers used for amplification (PCR) are the universal primer pair Cytochrome b consisting of: L14841 5'AAAGCTTCCATCCAACATCTCAGCATGAT GAAA-3' (Forward) and H15149.5'AAACTGCAGCCCCTCCAGAATGAT ATTTGTCCTCA-3' (Reverse)(Kocher *et al*., 1989). The DNA used as a template at the amplification stage was *W. leerii* DNA which had been previously isolated.

The PCR component used following Elvyra and Duryadi (2007) consisted of 2.5 µl of *W. leerii* sample DNA, 1 µl of primer (Forward and Reverse), 15.5 µl of dH_2O, 10X of 5 µl of Coral load, and 2X of 25 µl of Toptaq .

Predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing or primary attachment stage at 57.4°C for 1 minute, elongation stage at 72°C for 1 minute, final extinction at 72°C for 10 minutes. The PCR process was carried out for 35 cycles.

PCR product electrophoresis and sequencing

Visualization of the DNA bands of PCR products was carried out using the electrophoresis method as before. The electrophoresis results of a properly amplified PCR product will show one band without impurities. The PCR products were then sequenced. Sequencing was carried out by sending 50 µl of PCR results to PT. Genetics Science Indonesia, Jakarta.

Results

The total DNA molecule of *W. leerii* that had been extracted and isolated was then visualized using the electrophoretic method, 9 samples were selected from 30 samples (three samples per river) (Figure 1). DNA that has good quality will show DNA bands like lines.

The results of DNA products that have good quality are then amplified by the PCR method. The PCR amplification product uses the universal Cytochrome b gene with a different band thickness in each DNA (Figure 2).

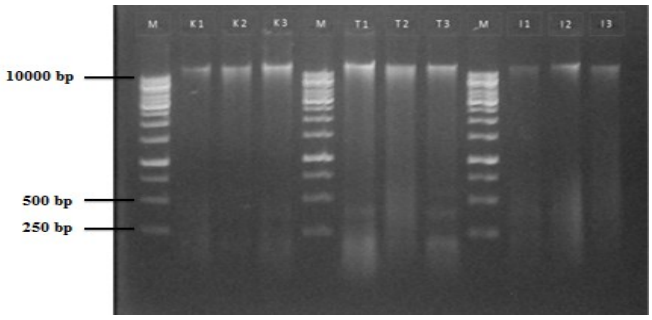

Figure 1. Total DNA profile of *W. leeri*. Information: (M) DNA ladder, (K1-K3) Samples of *W. leeri* from the Kampar River, (T1-T3) Samples of *W. leeri* from the Tapung River, (I1-I3) Samples of *W. leeri* from the Indragiri River.

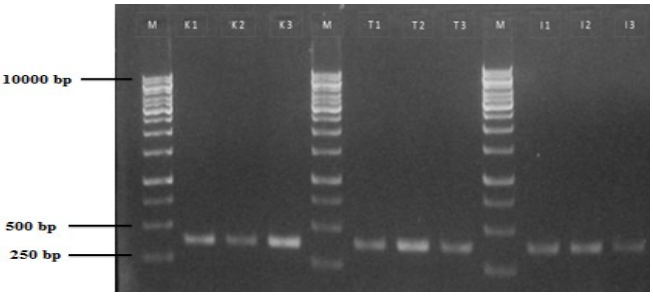

Figure 2. Results of DNA amplification of the Cytochrome b gene of *W. leeri*. Information: (M) DNA ladder, (K1-K3) Sample *W. leeri* from Kampar River, (T1-T3) Sample *W. leeri* from Sungai Tapung, (I1-I3) Sample *W. leeri* from Indragiri River.

Alignment was carried out using comparison species obtained from the results of BLASTn (Basic Local Alignment Search Tool nucleotide) in GenBank, namely *Wallago leerii* (Access code DQ119387.1), *Ompok pabo* (Access code FJ711274.1), *O. bimaculatus* (Access code KJ646881.1) and as an outgroup, namely *Silurus microdorsalis* (Access code DQ321756.1), the results of the phylogenetic tree construction are shown in Figure 3. The results of the alignment of the nucleotide bases of L14841 (F) and h15149 (R) in *W. leerii* DNA obtained fragment lengths of around 300-350 bp is then translated into amino acids. The amino acid sites obtained show the differentiation for the genus at the 10th site which is at the 28th, 29th and 30th nukeotides (Table 2).

Discussion

Cytochrome b gene amplification in the PCR process using mitochondrial DNA fragments from *W. leerii* obtained a sequence of 338 bp. Based on the length of the fragments obtained, the results were not much different referring to the research that had been carried out on *Kryptopterus limpok* and *K. apogon* from the Kampar and Indragiri Rivers using the universal Cytochrome b gene L14841 (F) and h15149 (R) obtained sequences of the same size 373 bp (Elvyra *et al.*, 2009). Other studies have revealed that Cytochrome b gene fragments reach 300-400 bp in fish (Kiril'Chik and Slobodyanyuk, 1997; Akasaki *et al.*, 2006; Hsieh *et al.*, 2010).

Based on the analysis of the genetic distance between *W. leerii* originating from the Kampar, Tapung and Indragiri rivers, the value is relatively small, ranging from 0.003–0.015 (Table 1). The genetic distance between *W. leerii* from the Kampar, Indagiri, Tapung rivers, and *W. leerii* from GenBank had a genetic distance of 0.015-0.018, 0.018-0.021, and 0.018-0.024. The genetic distance found in *W. leerii* intraspecies revealed that there were variations in the DNA structure that might have occurred due to random crossing over of genetic information in the fertilization process (Féral, 2002). The genetic distance that has the greatest value is between *O. bimaculatus* and *S. microdorsalis* 0.169. A genetic distance value > 0.03 indicates that one fish species is different from another, so intraspecies occurs if the genetic distance value is <0.03 (Muchlisin *et al.*, 2022; Nur *et al.*, 2022).

Based on the construction of the phylogenetic tree *W. leerii* from the Kampar, Tapung and Indragiri rivers and *W. leerii* comparator from GenBank formed one cluster and separated from *O. pabo*, *O. bimaculatus*, and *S. microdorsalis* which became an outgroup (Figure 3). The kinship in the first cluster, namely *W. leerii* from the Kampar, Tapung and Indragiri rivers with *W. leerii* from GenBank, has a bootstrap value of up to 100% (identical). In a separate outgroup cluster due to a different genus.

The 338 bp sequence was then translated using the MEGA version 6.0 program to produce 112 amino acid sites. Based on the site obtained, there is one genus coding site, namely at the 10th site position,

with the nucleotide composition there are sites 28th, 29th and 30th (Table 2).

Table 1. Genetic p-distance based on the Cytochrome b gene of *W. leeri*.

Individu	1	2	3	4	5	6	7	8	9	10	11	12	13
1													
2	0.009												
3	0.009	0.000											
4	0.003	0.006	0.006										
5	0.006	0.003	0.003	0.003									
6	0.012	0.009	0.009	0.009	0.006								
7	0.015	0.012	0.012	0.012	0.009	0.012							
8	0.003	0.006	0.006	0.006	0.003	0.009	0.012						
9	0.003	0.006	0.006	0.000	0.003	0.009	0.012	0.006					
10	0.021	0.018	0.018	0.018	0.015	0.015	0.024	0.018	0.018				
11	0.112	0.104	0.104	0.109	0.107	0.107	0.115	0.109	0.109	0.107			
12	0.115	0.109	0.109	0.112	0.112	0.112	0.121	0.115	0.112	0.107	0.101		
13	0.151	0.148	0.148	0.148	0.145	0.145	0.154	0.148	0.148	0.142	0.157	0.169	

Description: (1-3) *W. leerii* from Indragiri River, (4-6) *W. leerii* from Kampar River, (7-9) *W. leerii* from Tapung River, (10) *W. leerii*, (11) *O. pabo*, (12) *O. bimaculatus*, and (13) *S. Microdorsalis* from GenBank.

Table 2. Sites encoding the amino acid gene for Cytochrome b of *W. leerii*.

No.	The site of the amino acid codon	Accession number	10														
1	Wallago_leerii_RE_I1U	MH633741	L	G	S	L	L	L	L	C	L	L	M	Q	I		L
2	Wallago_leerii_RE_I2U	MH633742	F	.	.	.	.	.	.	.	.	.	.	.	.		
3	Wallago_leerii_RE_I3U	MH633743	F	.	.	.	.	.	.	.	.	.	.	.	.		.
4	Wallago_leerii_RE_K1U	MH633744	F	.	.	.	.	.	.	.	.	.	.	.	.		.
5	Wallago_leerii_RE_K2U	MH633745	F	.	.	.	.	.	.	.	.	.	.	.	.		.
6	Wallago_leerii_RE_K3U	MH633746	F	.	.	.	.	.	.	.	.	.	.	.	.		.
7	Wallago_leerii_RE_T1U	MH633747	F	.	L	.	.	.	.	.	.	.	.	.	.		.
8	Wallago_leerii_RE_T2U	MH633748	.	.	.	.	.	.	.	.	.	.	.	.	.		.
9	Wallago_leerii_RE_T3U	MH633749	F	.	.	.	.	.	.	.	.	.	.	.	.		.
10	Wallago_leerii	DQ119387.1	F	.	.	.	.	.	.	.	.	.	.	.	.		.
11	Ompok_pabo	FJ711274.1	F	.	.	.	.	.	.	.	.	I	.	.	.		.
12	Ompok_bimaculatus	KJ646881.1	F	.	.	.	.	.	.	.	.	I	.	.	.		.
13	Silurus microdorsalis	DQ321756.1	F	.	.	.	.	.	.	.	.	M	.	.	.		.

Description: (RE-I1U, RE-I2U, and RE-I3U) *W. leerii* from Indragiri River, (RE-K1U, RE-K2U, and RE-K3U) *W. leerii* from Kampar River, (RE-T1U, RE-T2U, and RE-T3U) *W. leerii* from Tapung River, (10) *W. leerii*, (11) *O. pabo*, (12) *O. bimaculatus*, and (13) *S. Microdorsalis* from GenBank.

The occurrence of transition substitution in the nucleotides of the Cytochrome b gene in *W. leerii* is presented in Table 3. Based on the values, it can be analyzed that transitional substitutions between *W. leerii* from the Kampar, Tapung and Indragiri rivers have relatively differences, ranging from 0–4 nucleotides. In *W. leerii* from the Kampar river and *W. leerii* from GenBank, there was a transitional substitution of 2–3 nucleotides, between *W. leerii* from the Indragiri, Tapung rivers, and *W. leerii* from GenBank, ranging from 3-4 nucleotides. The transitional substitution value that has the greatest value is between *O. pabo* and *S. microdorsalis* reach of 35 nucleotides.

The transversion substitution events in the nucleotides of the *W. leerii* Cytochrome b gene are presented in Table 4. The smallest transversion substitution value was 0, which means that no nucleotide transversion substitution occurred in some *W. leerii* DNA samples from Riau Province. Meanwhile, the biggest one is between *O. bimaculatus* and *S. microdorsalis* reaching 24 nucleotides.

W. leerii nucleotide transversions and substitutions cause variations in DNA structure. This allows fish to have variations in morphology, for example differences in color, number of fin rays, size and behavior (Hrbek and Larson, 1999; Jang et al., 2019). However, this variation does not cause the fish to become a separate species. It is evident from the phylogenetic tree which shows the monophylletic relationship between *W. leerii* collected from different locations.

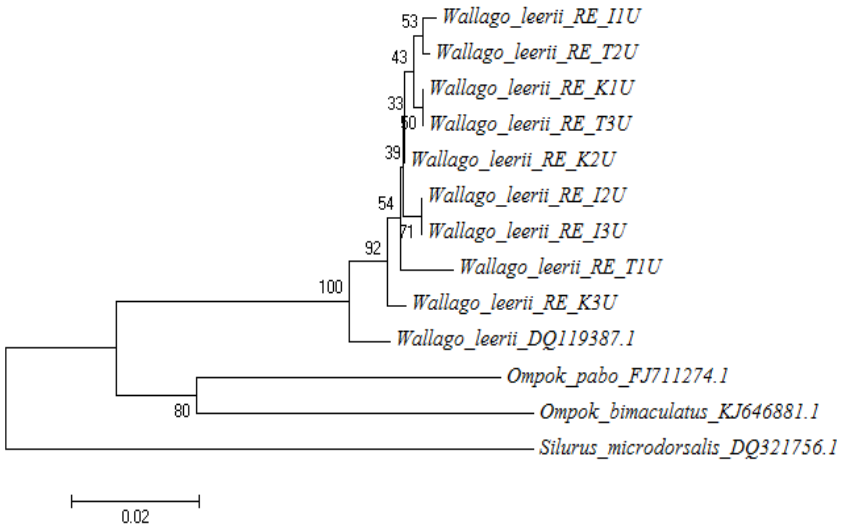

Figure 3. Phylogenetic tree construction using the neighbor-joining method and bootstrap analysis (1000 times

Table 3. Nucleotide base transition substitution of Cytochrome b gene of *W. leerii*

Individu	1	2	3	4	5	6	7	8	9	10	11	12	13
1													
2	3												
3	3	0											
4	1	2	2										
5	2	1	1	1									
6	3	2	2	2	1								
7	4	3	3	3	2	2							
8	1	2	2	2	1	2	3						
9	1	2	2	0	1	2	3	2					
10	4	3	3	3	2	3	4	3	3				
11	25	22	22	24	23	24	25	24	24	22			
12	28	26	26	27	27	28	29	28	27	26	24		
13	30	29	29	29	28	29	30	29	29	26	35	33	

Description: (1-3) *W. leerii* from Indragiri River, (4-6) *W. leerii* from Kampar River, (7-9) *W. leerii* from Tapung River, (10) *W. leerii*, (11) *O. pabo*, (12) *O. bimaculatus*, and (13) *S. Microdorsalis* from GenBank.

Table 4. Substitution of nucleotide base transversion of the Cytochrome b gene in *W. leerii*

Individu	1	2	3	4	5	6	7	8	9	10	11	12	13
1													
2	0												
3	0	0											
4	0	0	0										
5	0	0	0	0									
6	1	1	1	1	1								
7	1	1	1	1	1	2							
8	0	0	0	0	0	1	1						
9	0	0	0	0	0	1	1	0					
10	3	3	3	3	3	2	4	3					
11	13	13	13	13	13	12	14	13	13	14			
12	11	11	11	11	11	10	12	11	11	10	10		
13	21	21	21	21	21	20	22	21	21	22	18	24	

Description: (1-3) *W. leerii* from Indragiri River, (4-6) *W. leerii* from Kampar River, (7-9) *W. leerii* from Tapung River, (10) *W. leerii*, (11) *O. pabo*, (12) *O. bimaculatus*, and (13) *S. Microdorsalis* from GenBank.

Conclusion

The alignment of the nucleotides L14841 (F) and h15149 (R) in the 338 bp DNA of *W. leerii* was then translated into 112 amino acids. The amino acid sites obtained show a differentiator for the genus at the 10t^{h} site which is at the 28th, 29th, and 30th nukeotides. The construction of the phylogenetic tree of *W. leerii* f from Riau Province and *W. leerii* from GenBank formed one cluster.

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