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Genetic diversity analysis and phylogeography of *Osteochilus spilurus* (Cyprinidae: Labeoninae) from Bangka, Belitung, and Kalimantan Islands (Indonesia) using Cytochrome b gene

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Abstract. *Kurniawan A, Hariati AM, Kurniawan A, Kurniawan N, Nugroho TW, Bidayani E, Syarif AF, wiadnya DGR. 2022. Genetic diversity analysis and phylogeography of* Osteochilus spilurus *(Cyprinidae: Labeoninae) from Bangka, Belitung, and Kalimantan Islands (Indonesia) using Cytochrome b gene. Biodiversitas 23: 4738-4746.* The Sundaland fish species, *Osteochilus spilurus* is only utilized commercially in the Belitung Islands and has yet to be domesticated. This species can characterize freshwater fish phylogeography because have a minimum of human influence. This study aimed to analyze the genetic diversity and phylogeography of *O. spilurus* from the Bangka, Belitung, and Kalimantan Islands using Cytochrome b gene. Twenty fish samples were collected from the Sebangau and Kapuas Rivers in Kalimantan, the Lenggang River in Belitung Island, and the Lebak River in Bangka Island. Forward LA-cyp and reverse HA-cyp primers were used for amplification Cytochrome b gene. Mega 10.0 applications were used to construct of the phylogenetic tree. The phylogeographic analysis uses a comparative phylogenetic tree approach with geographical history. The cytochrome b gene showed variations in the nucleotides of *O. spilurus* from the three islands. Phylogeography of *O. spilurus* showed that there are two different clades with intraspecific variation according to a geographic area with bootstrap values of 99-100%. One clade contains fish samples from Bangka and Belitung islands, while the other clade comes from Kalimantan island. The formation of the Karimata Strait (between Bangka-Belitung and Kalimantan Islands) and the pattern of paleo rivers are predicted to influence the disparity.

Keywords: Cytochrome b gene, *Osteochilus spilurus***,** phylogeography, Sundaland

INTRODUCTION

As one of the mega biodiversity countries in the world, Indonesia has a diversity of fish, especially freshwater fish. The relationship between the Indonesian Islands triggers this diversity. There are more than 1200 native freshwater fish species to Indonesia, including 134 endemic species. The family Cyprinidae dominates freshwater fish in the Sunda Shelf (Hubert et al. 2015). The west area is connected to Sundaland (Kolanowska et al. 2016). The great potential of Indonesian native fish has left them unexplored.

One potential native fish from Bangka and Belitung Islands, part of the Sundaland, is *Osteochilus spilurus*. This species belongs to the family Cyprinidae and is called *Cempedik* fish in Belitung and *Kepaet* fish in Bangka. The distribution of this species is in the islands of Sumatra, Bangka, Belitung, Kalimantan, and Malaya Peninsula (GBIF 2021). It includes an economically important fish, especially for the people of East Belitung (Kurniawan et al. 2020a), but unnoticed by another region (Kurniawan et al. 2020b). According to trade and economic value in Belitung island, efforts have been made to domesticate this fish, which is currently reaching the first level (Kurniawan et al. 2019). Nowadays, this species was included in the least-concerned (LC) category on IUCN Redlist (Lumbantobing and Huckstorf 2020).

Morphological and genetic techniques were used to identify the species. According to Kurniawan et al. (2021a), fish from Belitung and Bangka islands were similar to the meristic character of *O. spilurus* described by Weber and Beaufort (1916) and Karnasuta (1993). It has a compressed body, tapered snout, dorsal height gradually higher before dorsal fin, number of scales along the lateral line is 28-30, soft rays of dorsal fin 12-13, and dorsal fin start from the 7th or 8th scale of the lateral line. The first molecular identification of this species in Bangka and Belitung Islands using partial Cyt b gene (408 bp) shows a high relationship at 99-100% (Kurniawan et al. 2021b).

Research on molecular identification was enhanced using the longer Cyt b gene (1141 bp). This length is a complete mitochondrial Cyt b sequence (Kawamura et al. 2014). This gene is also suitable for genetic characterization, geographic variation identification, and cyprinid fish diversity (Sati et al. 2015). Cyt b gene is also useful for studying the phylogeography of Cyprinidae

(Kamarudin and Esa 2009). This complete mitochondrial Cyt b is expected to provide more information about *O. spilurus* with more nucleotide sequences. Furthermore, the use of different genes in this research is a further identification of *O. spilurus* to ensure its identification and determine its relationship with genetic records from different regions and other species in the same genus.

The sampling range, which was previously only on the islands of Bangka and Belitung, was expanded by searching for fish samples to the island of Kalimantan in this current research. The holotypes and most specimens of this species are found in Kalimantan. This study aimed to analyze genetic diversity and phylogeography of *O. spilurus* from the Bangka, Belitung, and Kalimantan Islands using the Cytochrome B gene. Furthermore, it can be used to illustrate how the three islands of Sundaland were formerly connected by freshwater.

MATERIALS AND METHODS

Sample source

Fresh fish specimens were collected randomly from local fishermen that caught them from rivers in Bangka, Belitung, and Kalimantan islands (Table 1, Figure 1). The sample location was determined by fishing group information. The sample from Lenggang River, Belitung was obtained from local fishermen's catch using a fishing trap called *Sero*. The catch in Lebak River, Bangka uses active fishing gear in the form of a net. Fish samples from Sebangau River were caught using lift nets, while in Kapuas River, using traps.

Procedures

Sample preservation

Twenty individual fish with morphological characters for *O. spilurus* as the keys on Weber and de Beaufort's (1916) were randomly drawn as samples. All of them were prepared for DNA isolation. Fish samples were killed in cold water and preserved in 96% ethanol for molecular analysis. After being submerged in 96% ethanol for at least 24 hours and the sample was completely fixed with a color change, the fish was wrapped in tissue paper moistened with 96% ethanol and stored at-20°C (Kusuma et al. 2016). Research samples are stored at the Ichthyology Laboratory, Universitas Brawijaya, Malang, Indonesia.

DNA isolation

DNA isolation using PromegaÔ kit wizard and following the procedures implemented by Abinawanto et al. (2019).

Table 1. Sampling location of *Osteochilus spilurus*

Island	Location	River	No. of samples	Date	Coordinate
Bangka	Payabenua Village, Mendo Barat Subdistrict, Bangka District	Lebak	2	03rd March 2019	2°04'21" S 105°52'12" E
Belitung	Lintang Village, Gantung Subdistrict, East Belitung District	Lenggang	1	24th April 2019	2°55'09"S 108°06'35"E
Kalimantan	Kereng Bengkirai, Sebangau Subdistrict, Palangkaraya City	Sebangau	2	21st September 2020	2°17'39"S, 113°54'02"E
Kalimantan	Lawang Kamah Village, Timpah Subdistrict, Kapuas District	Kapuas	2	29th October 2020	1°37'00"S, 114°30'37"E

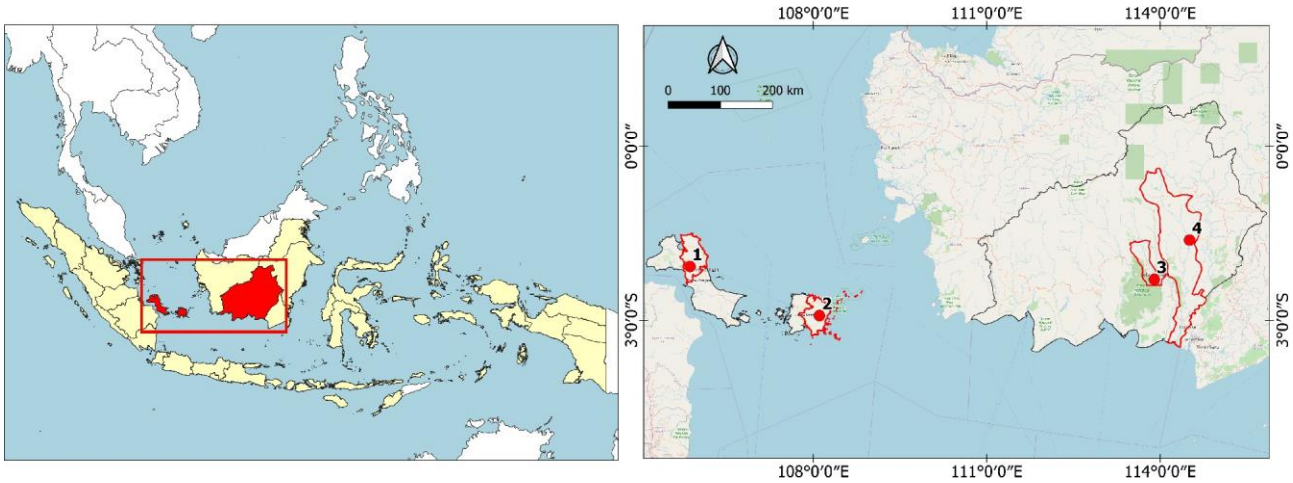

Figure 1. Sampling location of *Osteochilus spilurus*: 1. Lebak River, Bangka; 2. Lenggang River, Belitung; 3. Sebangau River, Central Kalimantan; and 4. Kapuas River, Central Kalimantan, Indonesia

The isolation process was initiated by inserting 500 µL of Nuclei Lysis solution and 120 µL of EDTA (0.5 M) pH 8 into a 1.5 mL microtube. The solution in the tube was cooled for ±10 minutes. Fin tissue and meat from fish samples were in sterile tubes and added 600 µL of a mixture of EDTA-Nuclei lysis and 17.5 µL of proteinase K. The mixture was incubated overnight at 55°C. The result of incubation was added 200 µL of protein precipitation solution and vortexed for 20 seconds, then the mixture was centrifuged for 4 minutes at a speed of 13,000-16,000 rpm. A pellet will appear at the bottom of the tube. The supernatant was transferred to a tube containing 600 µL of isopropanol and centrifuged for 1 minute at a speed of 13,000-16,000 rpm. The DNA pellet will appear white and the supernatant was removed, then 600 µL of 70% ethanol was added to the pellets and centrifuged for 1 minute at a speed of 13,000-16,000 rpm. The ethanol was removed and the pellets were aerated for 10 minutes. The pellets were added with 100 µL DNA rehydration solution and incubated at 65°C for 1 hour. Furthermore, DNA can be stored at a temperature of ± 4°C.

Amplification and sequencing

The purified total DNA was used as template DNA for amplification by PCR (Polymerase Chain Reaction). The PCR procedure requires 30 µL of each PCR tube, which is made up of 2 µL of template DNA, 11 µL of ddH_2O, 15 µL Go TaqÔ PCR mix, 1 µL of forwarding primer, and 1 µL of the reverse primer. The primers used were forward LA-cyp (5'-ATGGCAAGCCTACGAAAAAC-3') and reverse HA-cyp (5'-TCGGATTACAAGACCGATGCTT-3') (Yang et al. 2010). PCR setup based on Kenthao et al. (2016) as follows: pre-denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 45 seconds (denaturation), 58°C for 45 seconds (annealing), 72°C for 60 seconds (extension), 72°C for 7 minutes (post-extension), and soaking at 4°C.

The PCR results were tested qualitatively using electrophoresis to observe the bands that appeared under UV light. The 5 µL of PCR samples were mixed with 2 µL of loading buffer (30% glycerol, 0.2% bromphenol blue, 10 mM tris-HCL pH 8.0, and 0.1 mM EDTA). Then the mixture was put into 0.8% agarose gel (0.2 µg mL-1 SYBR SAFE) dissolved in TAE 50X (121 g Trizma, 28.6 mL glacial acetic acid, 0.5 M EDTA, and distilled water up to 500 mL). The samples were placed in the Garosa gel well, electrophoresis was carried out at 50V for 40 minutes in 1X TBE buffer (108 g Trizma, 55 g boric acid, 40 mL 0.5M EDTA, and up to 1 L distilled water). The positive results obtained when observed under UV light were the visualization of the total band of isolated DNA. Samples that show bands with positions according to markers in a qualitative test then read their nucleotide sequences in Applied Biosystems at the 1st Base, Malaysia.

Data analysis

A sequence that requires editing its electrogram for the primary nucleotide sequence and translated into the Chroma 2.6.6 program nucleotide sequence. Sequence results were compiled by forward and reverse sequence conventions using the Ugene 1.32 program. The consensus was aligned with other sequences using the Mesquite program. The comparison sequences used were sequences of the genus *Osteochilus* on the same gene and other sequences closely related to NCBI Blasts' results. Alignment of Cyt b gene sequences with LA-cyp and HA-cyp primers using comparison sequences from the species listed in Table 2 with *Labeo barbatulus* as outgroup. The results of the alignment of DNA sequences in phylogenetic analysis with Mega 10. A phylogenetic tree was constructed using the Kimura 2-parameter maximum likelihood model with 1000 bootstrap replications (Kimura 1980).

The phylogeographic analysis uses a comparative phylogenetic tree approach with geographical history. The position of the sampling location and the results of phylogenetic tree construction with geographic maps from Voris (2000) and ancient river maps (Hutama et al. 2016) to predict the relationship between geographic areas to genetic relationships. The phylogeographic analysis is presented descriptively.

RESULTS AND DISCUSSION

Morphological characters

Fish specimens from the four locations are true bony fish belonging to the order Cypriiformes, because they have a symmetrical head, body, and tail; have pelvic fins with the base located behind the median pectoral fins; pectoral fins are not spinous, and one dorsal fin. Fish belonging to the family Cyprinidae are characterized by forked pectoral-fin rays, 2 pairs of barbels around the mouth, bilateral flat bodies, and lateral lines. The genus *Osteochilus* is characterized by the form of hardened dorsal fins; anal-fin rays are not serrated and a dorsal fin has 10-18 weakly branched rays. These morphological characters are similar to the first specimens of *O. spilurus* from Bangka and Belitung (Kurniawan et al. 2021a).

Verification of morphological species in *O. spilurus* refers to Weber and Beaufort (1916) and Karnasuta (1993). All fish from all sampling locations had similar characteristics to the description keys. The samples were silvery color and turned brown when prepared using 96% ethanol. The body height increases from the snout to the beginning of the dorsal fin. A linea lateral line begins at the top of the operculum and ends in the middle of the base of the tail. The dorsal fin starts from the eighth scale in the line lateral. There is a black blotch at the base of the tail. The anal fin is behind the dorsal fin and the pelvic fin is behind the pectoral fin (Figure 2).

The number of scales along the lateral line on 29–30 scales is observed in samples from Bangka, Belitung, and Sebangau Rivers, whereas the number of scales in samples from Kapuas River is 30-31 scales. The number of scales from Kapuas River samples shows meristic variation with most previous studies on this species that describe 29-30 scales (Weber and Beaufort 1916), 28-29 scales (Karnasuta 1993), and 30 scales (Gunther 1868), but include the range of Inger and Kong (1962) description for sample from North Kalimantan is 29-32 scales with mean of 30.2.

Table 2. DNA sequences used in phylogenetic analysis using the cyt b gene

Species	Country	Locality	Cyt b gene length (bp)	GenBank Acc. number
Osteochilus hasselti	Indonesia[1]	Indonesia Market	402	KR007710.1
Osteochilus waandersii	Vietnam[1]	Dak Lak	577	MN541330.1
Osteochilus lini	Thailand[2]	-	1137	JX074265.1
Osteochilus sp.	Indonesia[1]	Baturaja Market, South Sumatra	1141	JX074233.1
Osteochilus vittatus	Kamboja[1]	Landing port, Kampong Chhnang	1141	JX074226.1
Osteochilus melanopleurus	Indonesia[1]	Siak River, Riau	857	MK036876.1
Osteochilus salsburyi	China[1]	Rong'an, Guangxi	1100	GU086539.1
Osteochilus spilurus	Indonesia[3]	Lenggang River, East Belitung	408	MT372794.1
Osteochilus spilurus	Indonesia[3]	Lenggang River, East Belitung	408	MT372795.1
Osteochilus spilurus	Indonesia[3]	Lebak River, Bangka	408	MT372796.1
Osteochilus spilurus	Indonesia[3]	Lebak River, Bangka	408	MT372797.1
Osteochilus spilurus	Indonesia*	Lenggang River, Belitung	1131	MZ146883
Osteochilus spilurus	Indonesia*	Lebak River, Bangka	1122	MZ146881
Osteochilus spilurus	Indonesia*	Lebak River, Bangka	1118	MZ146882
Osteochilus spilurus	Indonesia*	Sebangau River, Kalimantan	1158	MZ146884
Osteochilus spilurus	Indonesia*	Sebangau River, Kalimantan	1158	MZ146885
Osteochilus spilurus	Indonesia*	Kapuas River, Kalimantan	1158	MZ146876
Osteochilus spilurus	Indonesia*	Kapuas River, Kalimantan	1112	MZ146877
Labeo barbatulus	Laos[1]	-	1118	KC631289.1

Note: [1] NCBI (2022); [2]Yang et al. (2012); [3] Kurniawan et al. (2021); * Samples from this research

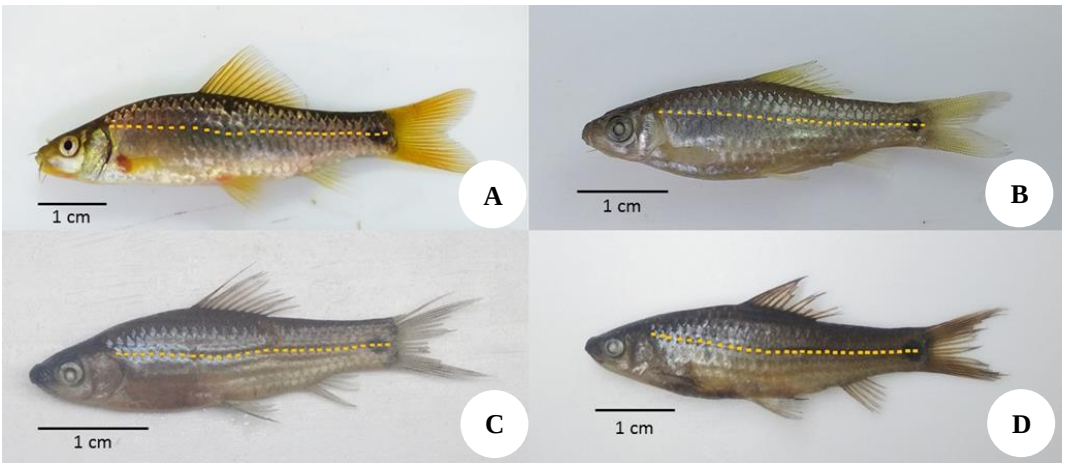

Figure 2. *Osteochilus spilurus* specimen used in the study from: A. Lebak river, Bangka; B. Lenggang river, Belitung; C. Sebangau river, Kalimantan; and D. Kapuas river, Kalimantan

Genetic diversity and phylogenetic analysis

Electrophoresis results showed the Cyt b DNA band was above 1000 bp (Figure 3). Most of the chromatograms from the sequencing results showed a normal pattern, but some sequences were judged to have overlapping patterns in the reverse section. Sequences were combined and produced nucleotide sequences with varying lengths ranging from 1118 to 1142 bp, while in other sequences, only forward nucleotide sequences were used (Table 3). The normal nucleotides produced are one from Belitung, and two each from Bangka, Palangkaraya, and Kapuas. One nucleotide from Belitung cannot be used to analyze because the nucleotide chromatogram sequencing results are not legible.

The nucleotide composition of *O. spilurus* from Bangka, Belitung, and Kalimantan is shown in Table 3. The lowest Guanine than other nucleotides also found in molecular identification of *O. spilurus* (Kurniawan et al. 2021b), *Cyclocheilichthys apogon* (Kenthao et al. 2016) using cyt b gene, *Osteochilus melanopleurus* (Asiah et al. 2020), *Labeo rohita, Catla catla, Cirrhinus mrigala, Labeo fimbriatus, Labeo bata,* and *Cirrhinus reba* (Mohanty et al. 2015) using COI gene. When compared to covalent bonds in A-T bases, C-G base hydrogen bonds are weak, making them easily disrupted and leading to alterations in the fundamental molecular structure of DNA (Astuti et al. 2020).

The nucleotide composition results in 326 variations for all islands. Variation becomes less when samples are grouped into Bangka-Belitung and Kalimantan (Tables 4 and 5). This high variation is correlated with a wide range of nucleotide compositions between sequences. When

sequences are grouped by sampling region, these nucleotide variations are less, indicating higher similarity. The same sequences from Kalimantan had 30 nucleotide variations, while the sequences from Bangka-Belitung showed 20 nucleotide variations. *Barbodes binotatus* from three different rivers in Java that are not connected to each other also show variations in guanine-cytosine and adenine-thymine. This difference causes genetic variation and shows the influence of geographical barriers and river flow on genetic drift (Astuti et al. 2020).

The blast sequence on the NCBI results shows that the sample has the closest relationship with *Bangana* sp., *Bangana dero, Labeo gonius*, and *L. barbatulus* with query coverage of more than 85%. That species has different morphological characteristics with samples. *Bangana dero* (Cyprinidae family) has a dorsal fin on the 10th predorsal scale and no more than 10 rays (Jadhav and Bano 2017). The genus *Labeo* has black blotch near the caudal fin, like the character of *O. spilurus*, but the number of scales on the lateral line is 44-51 (Sudasinghe et al. 2018). The absence of the same species in the Blast NCBI results allows the nucleotide record from this study to be one of the first records based on the Cyt b gene. Previous records that use the Cyt b gene 408 bp cause low query cover generated in the blasting process. The nucleotide record of the species *O. spilurus* based on the Cyt b gene with a length of more than 1000 bp complements the previous record using the cyt b gene 408 bp.

Based on phylogenetic analysis, the samples from Kalimantan and Bangka-Belitung separated to form different clades with 99% bootstrap (Figure 4). Samples from Bangka and Belitung islands are in the same clade with a strong bootstrap value (100%) and have a small genetic distance of 0-2.91% (Table 6). It is similar to the genetic identification of the same species using 408 bp cyt b, which show a genetic gap below 2% between Bangka and Belitung sample (Kurniawan et al. 2021b). While another clade formed from Sebangau and Kapuas River samples with strong bootstrap value. Nucleotides from different rivers in Kalimantan, which do not have connectivity with each other at present, also cannot be separated into different sub-clade with a genetic distance in the range of 1.09-5.57%. Samples in each clade are intraspecific in *O. spilurus* population. Nucleotides with genetic distance under 2% are included in intraspecific variation, but genetic variation between one and 2% indicates a population of the same species (Bradley and Baker 2001). Aminan et al. (2020) limited 3% genetic distances for intraspecific species. Some fish species like *Centropyge heraldi* and *Valenciennea wardii* have a maximum intraspecific distance of 5.92% dan *Scatophagus argus* at 6.52% (Steinke et al. 2009).

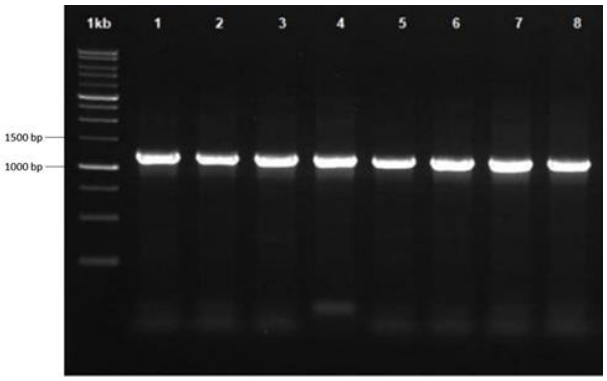

Figure 3. Electrophoresis result of *Osteochilus spilurus* samples from Bangka, Belitung and Kalimantan using Cyt b gene. Note: lanes 1-2 from Kapuas, lanes 3 and 8 from Belitung, lanes 4-5 from Palangkaraya, and lanes 6-7 from Bangka

Table 3. Nucleotide composition of DNA sequence of cyt b gene sample *Osteochilus spilurus*

DNA Sample	Nucleotide composition (%)				Total
	T	C	A	G	
Kapuas 1	29.4	26.7	29.9	14.0	1118
Kapuas 2	29.7	26.7	29.4	14.2	1119
Bangka 8	29.0	28.0	29.1	14.0	1122
Bangka 9	29.1	28.0	28.7	14.1	1117
Belitung 5	29.3	27.6	29.3	13.8	1120
Palangkaraya 1	29.5	26.8	29.9	13.8	1142
Palangkaraya 5	30.0	27.0	29.0	14.1	1138
Average	29.7	26.9	29.3	14.1	1124.4

Table 4. Nucleotide variation of the cyt b gene sequence from Kalimantan, Indonesia

	6	7	11	13	22	23	29	30	31	33	36	42	47	52	61	65	71	101	115	249	320	343	371	662	1065	1112	1137	1138	1139	1141
Kapuas1	C	A	A	T	G	T	A	C	G	A	T	A	C	A	C	T	T	G	G	A	G	C	A	C	C	A	N	N	N	N
Kapuas2	N	N	N	N	T	G	.	.	.	.	A	.	.	.	.	.	.	T	.	C	.	T	.	.	.	.	G	T	T	A
Palangkaraya1	A	C	.	A	T	.	.	.	.	.	A	.	.	.	T	.	A	T	A	C	A	.	.	.	T	G	T	A	G	T
Palangkaraya5	N	C	C	.	T	.	G	T	C	C	.	T	T	C	A	C	G	T	A	C	A	.	G	T	T	G	T	A	G	T

Table 5. Nucleotide variation of the cyt b gene sequence from Bangka and Belitung, Indonesia

	73	135	183	290	359	446	512	530	548	608	758	803	851	942	968	1040	1085	1088	1098	1147
Bangka 8	G	C	A	T	G	G	G	T	C	A	C	C	G	C	C	A	T	T	C	C
Bangka 9	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Belitung 5	A	T	G	C	A	A	A	C	T	G	T	T	A	A	T	G	C	A	T	T

Table 6. The genetic distance of *Osteochilus spilurus* with the comparison species in the Cyt b gene

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	0.0000																
2	0.0009	0.0000															
3	0.0181	0.0172	0.0000														
4	0.0262	0.0203	0.0086	0.0000													
5	0.0262	0.0203	0.0086	0.0000	0.0000												
6	0.0115	0.0115	0.0233	0.0137	0.0137	0.0000											
7	0.0291	0.0233	0.0115	0.0027	0.0027	0.0165	0.0000										
8	0.1842	0.1814	0.1774	0.1606	0.1606	0.1713	0.1641	0.0000									
9	0.2418	0.2389	0.2344	0.3630	0.3630	0.3788	0.3682	0.0555	0.0000								
10	0.1999	0.1968	0.1927	0.2832	0.2832	0.2967	0.2876	0.0356	0.0557	0.0000							
11	0.1819	0.1799	0.1759	0.1481	0.1481	0.1589	0.1516	0.0109	0.0596	0.0233	0.0000						
12	0.2091	0.2098	0.2060	0.2017	0.2017	0.2017	0.2054	0.2704	0.4997	0.3840	0.2432	0.0000					
13	0.2358	0.2339	0.2249	0.1723	0.1723	0.1795	0.1759	0.2229	0.2842	0.2640	0.2177	0.1586	0.0000				
14	0.2452	0.2406	0.2355	0.2092	0.2092	0.2206	0.2130	0.2129	0.2781	0.2518	0.2063	0.1475	0.1138	0.0000			
15	0.1994	0.1976	0.1917	0.1795	0.1795	0.1796	0.1832	0.2087	0.2630	0.2388	0.1973	0.1152	0.1662	0.1734	0.0000		
16	0.2244	0.2239	0.2138	0.2017	0.2017	0.2018	0.2055	0.2398	0.2936	0.2698	0.2283	0.1320	0.1734	0.1768	0.0906	0.0000	
17	0.2179	0.2196	0.2142	0.1886	0.1886	0.1886	0.1944	0.2302	0.2285	0.2322	0.2302	0.1136	0.1830	0.1775	0.1382	0,1729	0.0000

Note: 1. Bangka 8; 2. Bangka 9; 3. East Belitung 5; 4. MT372794.1; 5. MT372795.1; 6. MT372796.1; 7. MT372797.1; 8. Palangkaraya 1; 9. Palangkaraya 5; 10. Kapuas 1; 11. Kapuas 2; 12. *O. spilurus* Sabah); 13. *O. waandersii*; 14. *O. lini*; 15. *Osteochilus* sp.; 16. *O. vittatus*; 17. *O. melanopleurus* (Table 2 and Figure 4)

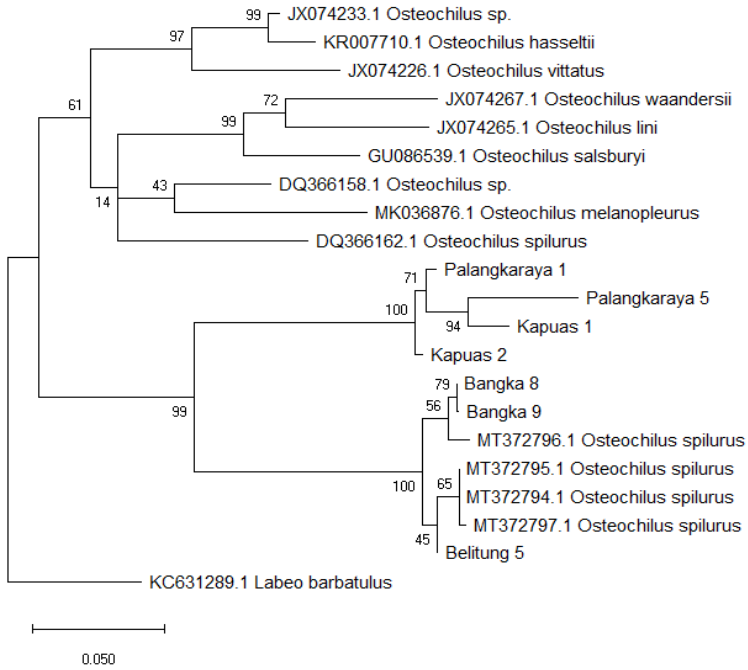

Figure 4. Phylogenetic tree of *Osteochilus spilurus* and closely related species based on the cyt b gene (1141 bp) maximum-likelihood method with 1000 replication. *Labeo barbatulus* from the family Cyprinidae was used as an outgroup

Phylogeography analysis

Bangka Belitung belongs to a distinct clade than Palangkaraya and Kapuas. The different clades allow for the contribution of regional isolation to genetic diversity. The sea can represent geographic barriers to genetic diversity (Zhang et al. 2011). Gaspar strait between Bangka and Belitung Islands that predict developed 6000 years ago (Voris 2000), should be made a geographical barrier but not affect to genetic variation of *O. spilurus*. Similar condition occurs in the complex species *Rasbora lateristriata* in Bali, Lombok, and Sumbawa, which are in the same sub-clade even though they are separated on three different islands (Kusuma et al. 2016). Furthermore, Kusuma et al. (2016) predict human factors affect the genetic connection. It possibly happens in Bangka and Belitung, although there is no indication of the human-caused distribution of this fish due to the lack of attention to it outside East Belitung (Kurniawan et al. 2020c). Different river separations did not also cause genetic diversity in Kalimantan samples. They can create genetic links because they live in the same region and on the same island.

Bangka-Belitung and Kalimantan are separated by 17.59-24.18% genetic distance (Table 6). This distance resulted in a separate clade on the phylogenetic tree for both regions divided by the Karimata Straits. According to Voris (2000), the split of Bangka-Belitung and Kalimantan at the Karimata Strait happened 10,000 years ago (Figure 5A). This physical isolation may have affected genetic diversity. *Hemibagrus nemurus* and *H. wyckioides* from Sumatra, Java, and Kalimantan were in the different clades in phylogenetics using Cyt b (Megarani et al. 2020). The circumstance allows cryptic species to exist among them. Genetic distances of up to 12.3% as a cryptic species on *Rasbora adisi* (Sudasinghe et al. 2020). Bali Strait, which separated Java and Bali Islands formed in the same period as the Karimata Strait (Figure 5A), was predicted to cause cryptic species *Rasbora lateristriata* from the Java and Bali islands (Kusuma et al. 2016).

According to Kusuma et al. (2016), the cryptic species *Rasbora lateristriata* was thought to exist because of a geological barrier and it was found in the same paleo river. *Osteochilus spilurus* may also have been affected by variations in former paleo river flows. The Sebangau and Kapuas rivers in Central Kalimantan are connected to the East Sunda ancient river, which empties into the East Java Sea. Meanwhile, the North Sunda paleo river empties into the Natuna Sea is more relevant to Bangka and Belitung (Figure 5B). Similar findings are found in the phylogeography of *Scleropages formosus* and *S. inscriptus*, which show variations related to the paleo-rivers pattern. Asian Arowana (*Scleropages* spp.) from Central, East, and South Kalimantan differ from those in West Kalimantan and southern Sumatra (Alshari et al. 2021). Tigerfish (*Datnioides* sp.) from West Kalimantan and Sumatra also belong to the same lineage as the ancient river pattern (Fahmi et al. 2018). The North Sunda Paleo River connects Sumatra's Indragiri, Hari (Batang Hari), and Musi rivers with West Kalimantan's Kapuas River and drains into the Natuna Islands. On the other hand, the East Sunda Paleo River runs into the Java Sea and connects to rivers in Borneo's southern region (Hutama et al. 2016).

A **B**

Figure 5. Distribution of *Osteochilus spilurus* in Indonesia: (A) the formation of Karimata Strait 10,000 years ago and (B) Sundaland 21,000 years ago (Irwanto 2019) with prediction area of Nort Sunda (white dotted line) and East Sunda (yellow dotted line) ancient river (Hutama 2016). Location of *O. spilurus* population in Bangka (1), East Belitung (2), Palangkaraya (3), and Kapuas (4)

In conclusion, a comparative phylogenetic tree technique combined with geographic history resulted in nucleotide variations of *O. spilurus* from the three separate islands using the cytochrome b gene. This phylogeography revealed two distinct clades with intraspecific variation based on geographic location, with bootstrap values of 99-100%. Fish samples from Kalimantan Island make up one clade, while those from Bangka and Belitung Islands make up the other. The formation of the Karimata Strait (between the islands of Bangka-Belitung and Kalimantan) and the pattern of paleo rivers are predicted to impact the discrepancy.

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