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Phylogeographic Connectivity of *Osteochilus spilurus* (Cyprinidae) from Bangka, Belitung, and Kalimantan Islands Using DNA Barcoding

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ABSTRACT

Osteochilus spilurus (Bleeker, 1851), with a vast distribution in Sundaland, is one of the least utilized freshwater species. The lack of interest in this species has resulted in restricted human-caused dispersal and has offered the opportunity for a precise phylogeographic study. Fish samples were caught from six rivers on the islands of Bangka, Belitung, and Kalimantan. Fish identification was conducted using DNA barcoding of cytochrome oxidase 1 with Fish-F1 and Fish-R1 primers. Twenty-five 655 bp nucleotide sequences from this research are recorded in NCBI and represent the first such records. Bangka-Belitung and Kalimantan samples form intraspecific relationships within each region but are separated into different clades between the two regions. This separation suggests that *O. spilurus* may represent a cryptic species due to historical changes in the formation of the Karimata Strait between the two regions. *O. spilurus* has the potential to be used as a distribution and phylogeographic model for freshwater fish in Sundaland.

INTRODUCTION

The separation by the ocean due to interglacial processes causes geographic isolation of the islands. Such conditions allow the emergence of genetic variations of fish in the same species due to differences in environmental adaptation and the disconnection of reproductive relations with the same species in other areas. Freshwater fish that are geographically separated for a long time allow for differences in the adaptation process to the environment which results in changes in their morphology. Fish with separate or isolated populations can experience changes in morphological characters and it is indicated that an initial speciation process has occurred in the form of the formation of fish subspecies **(Budiharjo, 2001)**. Differences in geographical areas in the islands allow for variations in fish classification **(Itsukushima, 2019)**. Historical biogeography is formed by the isolation that occurs due to sea level rise.

Freshwater fish is the most effective fauna to explain biogeography because of its limited ability to spread to other areas. Freshwater fish that have a wide distribution and have not been affected by the role of humans in their distribution can provide an overview of their historical biogeographic patterns. One of the potential freshwater fish is *Osteochilus spilurus* (Bleeker, 1851). This species is only popular and consumed on Belitung Island **(Kurniawan & Triswiyana, 2019; Kurniawan *et al*., 2019)**, but there is no indication of its use in Sumatra, Bangka, and Kalimantan **(Pramono *et al*., 2020)**. *O. spilurus* is also a blackwater fish that is resistant to waters with low pH **(Fahmi *et al*., 2015; Sule *et al*., 2016; Kurniawan *et al*., 2020b)**. In fact, this species is reported as the most abundant fish species in Kalimantan peatlands **(Haryono, 2012)**. Fish with this ability are categorized as extremophile fish **(Kurniawan & Mustikasari, 2021; Kurniawan *et al*., 2024)**. The minimal possible role of human distribution and its resistance to extreme waters makes this species suitable for use in phylogeographic studies that can underlie biogeographic studies.

O. spilurus has been studied for its function of describing the geographical distribution pattern of freshwater fish in Sundaland using the cytochrome b gene. The genetic variation that forms clade differences in phylogeography is not only caused by the geographical isolation of island formation but is also predicted to be influenced by ancient river patterns **(Kurniawan *et al*., 2022)**. These findings need to be confirmed by phylogeographic studies using the cytochrome oxidase 1 (*CO1*) gene as well as adding to the DNA barcode database for *O. spilurus*. *CO1* gene is the recommended gene in fish barcoding **(Hubert *et al*., 2003)**. The co1 gene is also applied in genetic identification to ensure accurate fish species determination **(Syarif *et al*., 2025b)**.

MATERIALS AND METHODS

This DNA barcoding and phylogeographic research of *O. spilurus* was carried out from March 2020 to August 2022. Sampling of fish on the islands of Bangka and Belitung was conducted through direct exploration. Meanwhile, fish from Kalimantan were obtained using local field technical personnel and sent via expeditions.

Sampling and sample processing

Fresh fish samples were randomly collected from local fishermen who caught them in rivers on the islands of Bangka, Belitung, and Kalimantan (Table 1 & Fig. 1). A fishing trap known as a *Sero* was used to collect samples from the Lenggang River on Belitung Island. A net was used as active fishing equipment to catch fish in the Lebak River and Bencah Village on Bangka Island. Lift nets were used to catch fish samples in the Sebangau and Katingan rivers, while traps were used in the Kapuas River. Fish samples were euthanized in cold water and preserved in 96% alcohol. After being submerged in 96%

alcohol for at least 24 hours, and once the sample was completely fixed as indicated by a change in color, the fish were wrapped in tissue paper moistened with 96% alcohol and stored at -20°C (**Kusuma *et al*.., 2016**).

Table 1. Sampling location of *O. spilurus*

No	Island	Location	River	Coordinate
1	Bangka	Payabenua Village, Mendo Barat District, Bangka Regency	Lebak	2°04'21" S 105°52'12" E
2	Bangka	Bencah Village, Air Gegas District, South Bangka Regency	Palm plantations irrigation	2º46'03" S, 106º23'48" E
3	Belitung	Lintang Village, Gantung District, East Belitung Regency	Lenggang	2°55'09"S 108°06'35"E
4	Kalimantan	Tumbang Linting Village, Katingan Hilir District, Katingan Regency	Tributary Katingan	1°57'08"S, 113°22'07"E
5	Kalimantan	Kereng Bengkirai, Sebangau District, Palangkaraya City	Sebangau	2°17'39"S, 113°54'02"E
6	Kalimantan	Lawang Kamah Village, Timpah District, Kapuas Regency	Kapuas	1°37'00"S, 114°30'37"E

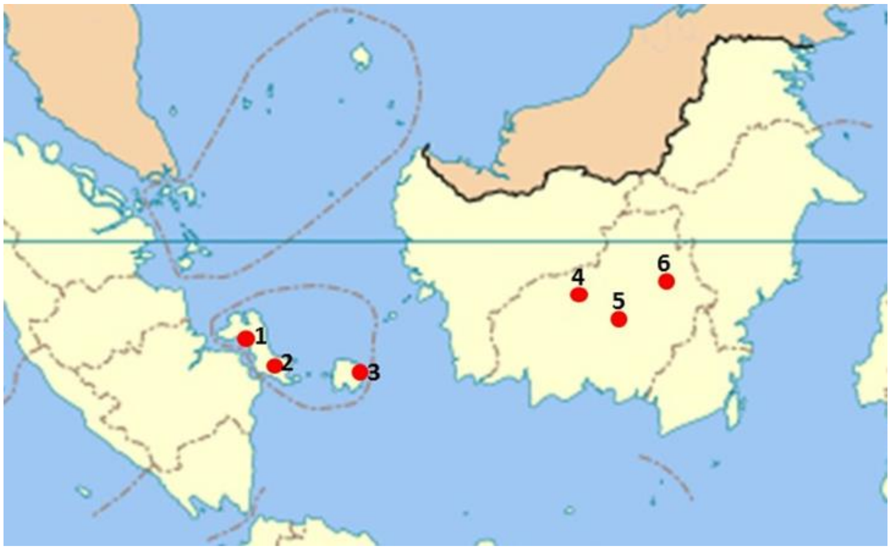

Fig. 1. Maps of sampling location of *O. spilurus.*
1) Bangka Regency, 2) South Bangka Regency, 3) East Belitung Regency, 4) Katingan Regency, 5) Palangkaraya City, 6) Kapuas Regency

DNA isolation and sequencing

The Wizard Promega™ kit was utilized in this work to isolate DNA. A 1.5ml microtube was filled with 500µl of nuclei lysis solution and 120µl of EDTA (0.5M, pH 8) to begin the isolation process. The solution in the tube was cooled for ten minutes. Fish fin and flesh tissue samples were placed in sterile tubes with 600µl of the EDTA–Nuclei Lysis Solution and 17.5µl of proteinase K. The mixture was incubated overnight at 55°C. Following incubation, 200µl of protein precipitation solution was added, and the mixture was vortexed for 20 seconds. It was then centrifuged at a speed between 13,235 and 20,059 × *g* for 4 minutes, resulting in a pellet at the bottom of the tube.

The supernatant was transferred to a new tube containing 600µl of isopropanol and centrifuged for one minute at the same speed range. The supernatant was discarded, and a white DNA pellet was observed. A total of 600µl of 70% ethanol was added, followed by centrifugation for one minute at the same speed to produce pellets. After ethanol removal, the pellets were air-dried for 10 minutes. Then, 100µl of DNA rehydration solution was added, and the tubes were incubated at 65°C for one hour. The DNA can be stored at 4°C.

The purified total DNA was used as template DNA for amplification using the PCR technique. The PCR mix consisted of 2% forward and reverse primers, 50% PCR mix, 36% ddH$_2$O, and 10% template DNA. The primers used were FishF1 5’-TCAACCAACCACAAAGACATTGGCAC-3’ (forward) and FishR1 5’-TAGACTTCTGGGTGGCCAAAGAATCA-3’ (reverse) **(Ward *et al.*, 2005)**. The PCR settings were as follows: pre-PCR (94°C for five minutes), denaturation (94°C for 30 seconds), annealing (52°C for 30 seconds), extension (72°C for 30 seconds), and post-PCR (72°C for five minutes), run for 35 cycles. The nucleotide sequences of the PCR products were then analyzed using an Applied Biosystems platform at 1st BASE.

Table 2. Sequences compared on the *co1* gene

Species	Country	Location	Accession Number
Osteochilus vittatus	Indonesia	Rawa Pening, Ambarawa	KU692714.1
Osteochilus salsburyi	Cina		MH500668.1
Osteochilus salsburyi	Cina	Hekou, Province Yunnan[1]	GU086577.1
Squalidus chankaensis	GenBank		JN003374.1
Squalidus japonicus	GenBank		FJ197678.1
Osteochilus waandersii	Aquarium[2]		JX074190.1
Labiobarbus lineatus	China	Mengna, Yunnan	HM536885.1
Osteochilus lini	Thailand		JX074188.1
Osteochilus sp.	Indonesia	Batraja, South Sumatera	JX074151.1
Labeo altivelis	Congo	Odzala[2]	JX074218.1

Labeo altivelis	Congo	Aruwimi River	KT193284.1
Labeo weeksii	Congo	Kinshasa	HM418181.1

[1]**Zheng *et al.* (2010),** [2] **Yang *et al.* (2012)**.

Phylogenetic contruction

Sequences were initially trimmed using the Chromas 2.6.6 application. Forward and reverse sequences were assembled into consensus sequences using the UGENE 1.32 program. The resulting consensus sequences were aligned with other sequences using the Mesquite program. The aligned sequences are presented in Table (2), with *Squalidus chankaensis* and *Squalidus japonicus* used as outgroups.

The phylogenetic tree was constructed using the MEGA X application, applying the Maximum Likelihood method with the Kimura 2-parameter model and 1000 bootstrap replications **(Kimura, 1980)**.

RESULTS

DNA barcode record

All sequencing results of fish samples for the *CO1* gene showed normal chromatogram patterns and could be successfully combined between forward and reverse sequences. All combined sequences contained 655 bp of nucleotides. Twenty-five sequences from this research have been submitted to NCBI (Table 3).

Table 3. List of sequences record and accession numbers of *O. spilurus* from this research in NCBI

Number	Specimen Origin	Count of Sequence	Accession Number
1	Payabenua, Bangka, Bangka Island	5	MZ145235 - MZ145239
2	Katingan, Central Kalimantan	2	MZ144009 - MZ144010
3	Lawang Kamah, Kapuas, Central Kalimantan	4	MZ144005 - MZ144008
4	Kereng Bengkirai, Palangkaraya, Central Kalimanatan	3	MZ144002 - MZ144004
5	Air Gegas, South Bangka, Bangka Island	1	MZ144001
6	Gantung, East Belitung, Belitung Island	10	MZ143990 - MZ143999
	Jumlah	25	

Nucleotides composition

The sequences from Bangka Belitung consisted of thymine (29.6–29.9%), cytosine (26.1–26.4%), adenine (24.9–25.5%), and guanine (18.4–19.1%), with variations in 13 nucleotides (Table 4). Meanwhile, the sequences from Kalimantan consisted of thymine (28.9–29.2%), cytosine (26.4–26.7%), adenine (25.5–26.0%), and guanine (18.6–18.8%), with variations in 10 nucleotides (Table 5).

Table 4. Nucleotide composition of DNA sequences for *CO1* gene from Bangka-Belitung

Sample Code	Nucleotide Composition				Total
	T	C	A	G	
East Belitung 1	29.8	26.3	25.5	18.5	655
East Belitung 5	29.9	26.2	25.5	18.4	655
East Belitung 6	29.6	26.4	24.9	19.1	655
East Belitung 7	29.8	26.3	24.9	19.1	655
East Belitung 8	29.6	26.1	25.2	19.1	655
East Belitung 9	29.8	26.3	25.5	18.5	655
East Belitung 10	29.8	26.3	24.9	19.1	655
East Belitung 12	29.8	26.3	25.5	18.5	655
East Belitung 13	29.8	26.3	25.5	18.5	655
East Belitung 14	29.8	26.3	25.5	18.5	655
Bangka 1	29.8	26.3	25.5	18.5	655
Bangka 2	29.8	26.3	25.5	18.5	655
Bangka 3	29.8	26.3	25.5	18.5	655
Bangka 4	29.8	26.3	25.5	18.5	655
Bangka 5	29.8	26.3	25.5	18.5	655
Bangka 9	29.8	26.3	24.9	19.1	655
South Bangka 1	29.8	26.3	25.2	18.8	655
Average	29.8	26.3	25.3	18.7	655

Table 5. Nucleotide composition of DNA sequences of *CO1* gene from Kalimantan

Sample Code	Nucleotide Composition				Total
	T	C	A	G	
Palangkaraya 1	29.0	26.7	25.6	18.6	655
Palangkaraya 2	29.0	26.7	25.6	18.6	655
Palangkaraya 3	29.2	26.6	25.5	18.8	655
Kapuas 1	29.2	26.6	25.5	18.8	655
Kapuas 2	29.2	26.6	25.5	18.8	655
Kapuas 3	29.2	26.6	25.5	18.8	655
Kapuas 7	29.2	26.4	25.8	18.6	655
Katingan 5	28.9	26.6	26.0	18.6	655
Katingan 8	29.2	26.6	25.6	18.6	655
Average	29.1	26.6	25.6	18.7	655

Sequences from Bangka-Belitung and Kalimantan showed genetic variation across 326 nucleotides, and therefore are presented separately for Bangka-Belitung and Kalimantan (Tables 6, 7). Bangka and Belitung showed 13 nucleotide variations. Four samples from Belitung and five from Bangka had 100% nucleotide similarity. Sequences from Kalimantan showed 10 nucleotide variations.

Table 6. Nucleotide variation of samples from Bangka and Belitung on the *CO1* gene

Sample Code	Nucleotide Sequence												
	11	47	57	80	149	164	173	314	341	384	407	431	581
East Belitung 1	T	T	C	A	T	A	A	A	A	C	G	A	G
East Belitung 5	.	.	.	.	.	.	.	.	.	.	.	.	.
East Belitung 6	.	C	.	G	C	G	G	G	G	T	A	G	A
East Belitung 7	.	.	.	G	C	G	G	G	G	T	A	G	A
East Belitung 8	A	.	A	G	C	G	G	G	G	T	A	G	A
East Belitung 10	.	.	.	G	C	G	G	G	G	T	A	G	A
East Belitung 9	.	.	.	.	.	.	.	.	.	.	.	.	.
East Belitung 12	.	.	.	.	.	.	.	.	.	.	.	.	.
East Belitung 13	.	.	.	.	.	.	.	.	.	.	.	.	.
East Belitung 14	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 1	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 2	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 3	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 4	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 5	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangka 9	.	.	.	G	C	G	G	G	G	T	A	G	A
South Bangka 1	.	.	.	.	C	.	.	.	G	T	.	G	.

Table 7. Nucleotide variation of samples from Kalimantan on the *CO1* gene

Sample Code	Nucleotide Sequence									
	48	182	188	215	266	308	311	314	458	506
Palangkaraya 1	C	A	C	A	G	G	T	A	C	T
Palangkaraya 2	.	.	.	.	.	.	.	.	.	.
Palangkaraya 3	.	G	T	G	A	A	C	G	T	.
Kapuas 1	.	G	T	G	A	A	C	G	T	.
Kapuas 2	.	G	T	G	A	A	C	G	T	.
Kapuas 3	.	.	T	G	A	A	C	G	T	.
Kapuas 7	A	.	T	G	.	A	C	.	T	.
Katingan 5	.	.	A	G	.	A	C	.	T	A
Katingan 8	.	.	T	G	.	A	C	.	T	.

Phylogenetic construction

The results of the phylogenetic analysis of the *CO1* gene sequence from *O. spilurus*, compared with other species from the genus *Osteochilus* and several additional species, showed that *O. spilurus* does not yet have a closely related comparison gene. As shown in the phylogenetic tree, the sequence of *O. spilurus* forms a distinct clade, although it branches within the same larger group as the genus *Osteochilus*.

The phylogenetic tree shows that sequences from Kalimantan and Bangka-Belitung are separated into different branches (Fig. 2). The nine sequences from Kalimantan, which originate from different rivers, show 99% similarity. Despite the Kapuas, Sebangau, and Katingan rivers having different, unconnected channels, this is not clearly reflected in the phylogenetic branching. Similarly, samples from Bangka-Belitung, despite being separated by the ocean, also show 99% similarity among the 17 sequences (Table 7). The separation of sampling locations across different islands and the Gaspar Strait did not result in distinct phylogenetic branches due to low genetic distance. Some sequences from Bangka are grouped with those from Belitung, and vice versa.

Overall, the Bangka-Belitung group forms a distinct clade, separate from Kalimantan. *Squalidus chankaensis* and *Squalidus japonicus*, identified as the most similar species through BLAST analysis, were used as outgroups in the phylogenetic tree, showing a bootstrap value of 43% relative to the genus *Osteochilus.*

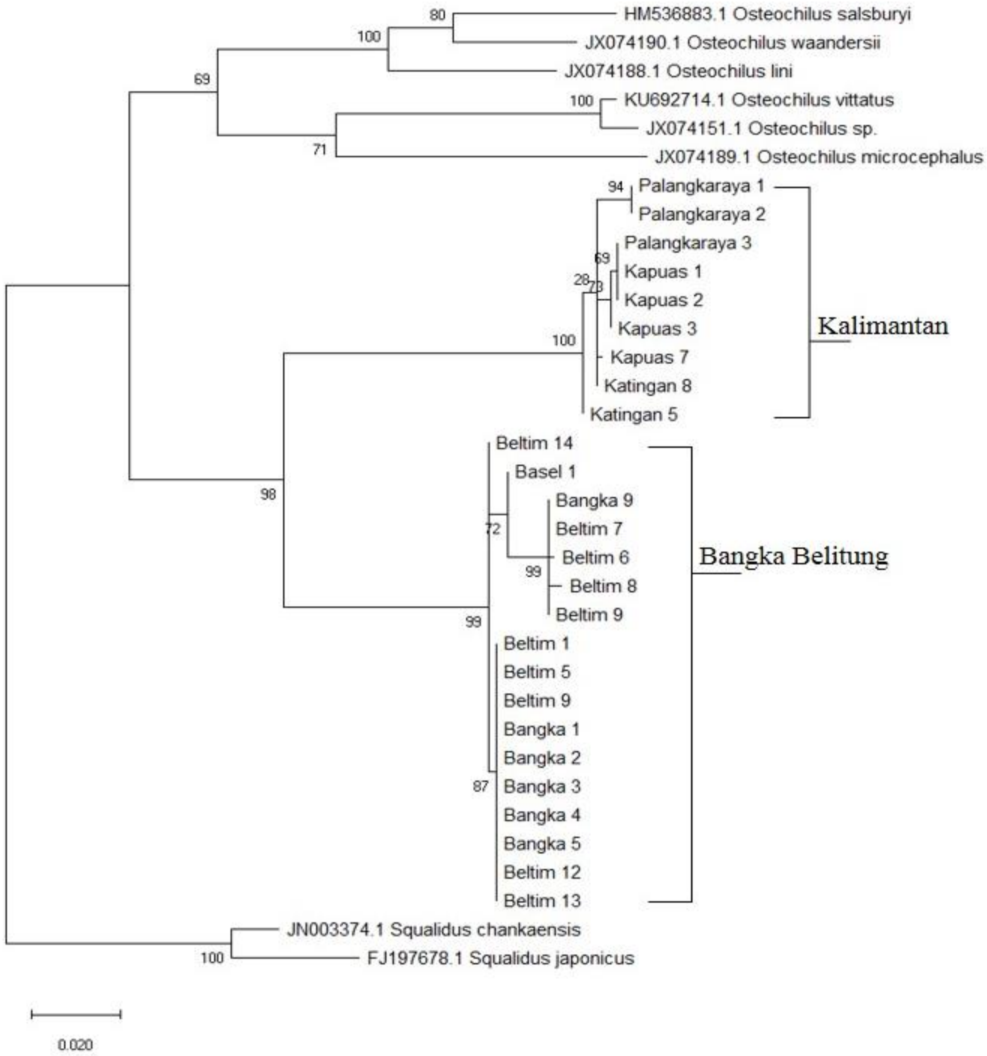

Fig. 2. Phylogenetic tree of *O. spilurus* on the *CO1* gene (Beltim is code of East Belitung)

Table 8. Genetic distance of *O. spilurus* in the *CO1* gene with comparison sequences

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
1																														
2	0.0000																													
3	0.0000	0.0000																												
4	0.0000	0.0000	0.0000																											
5	0.0000	0.0000	0.0000	0.0000																										
6	0.0114	0.0114	0.0114	0.0114	0.0114																									
7	0.0045	0.0045	0.0045	0.0045	0.0045	0.0068																								
8	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0045																							
9	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0045	0.0000																						
10	0.0125	0.0125	0.0125	0.0125	0.0125	0.0011	0.0079	0.0125	0.0125																					
11	0.0114	0.0114	0.0114	0.0114	0.0114	0.0000	0.0068	0.0114	0.0114	0.0011																				
12	0.0137	0.0137	0.0137	0.0137	0.0137	0.0023	0.0091	0.0137	0.0137	0.0034	0.0023																			
13	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0045	0.0000	0.0000	0.0125	0.0114	0.0137																		
14	0.0114	0.0114	0.0114	0.0114	0.0114	0.0000	0.0068	0.0114	0.0114	0.0011	0.0000	0.0023	0.0114																	
15	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0045	0.0000	0.0000	0.0125	0.0114	0.0137	0.0000	0.0114																
16	0.0000	0.0000	0.0000	0.0000	0.0000	0.0114	0.0045	0.0000	0.0000	0.0125	0.0114	0.0137	0.0000	0.0114	0.0000															
17	0.0000	0.0000	0.0000	0.0000	0.0000	0.0108	0.0027	0.0000	0.0000	0.0135	0.0108	0.0164	0.0000	0.0108	0.0000	0.0000														
18	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0883	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0569													
19	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0883	0.0869	0.0869	0.0869	0.0869	0.0869	0.0869	0.0601	0.0023												
20	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0884	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0572	0.0068	0.0057											
21	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0884	0.0870	0.0870	0.0870	0.0870	0.0870	0.0870	0.0572	0.0068	0.0057	0.0000										
22	0.0882	0.0882	0.0882	0.0882	0.0882	0.0856	0.0882	0.0882	0.0882	0.0869	0.0856	0.0856	0.0882	0.0856	0.0882	0.0882	0.0601	0.0057	0.0034	0.0091	0.0091									
23	0.0882	0.0882	0.0882	0.0882	0.0882	0.0856	0.0882	0.0882	0.0882	0.0869	0.0856	0.0856	0.0882	0.0856	0.0882	0.0882	0.0601	0.0057	0.0034	0.0091	0.0091	0.0000								
24	0.0882	0.0882	0.0882	0.0882	0.0882	0.0856	0.0882	0.0882	0.0882	0.0869	0.0856	0.0856	0.0882	0.0856	0.0882	0.0882	0.0601	0.0057	0.0034	0.0091	0.0091	0.0000	0.0000							
25	0.0869	0.0869	0.0869	0.0869	0.0869	0.0842	0.0869	0.0869	0.0869	0.0856	0.0842	0.0842	0.0869	0.0842	0.0869	0.0869	0.0572	0.0045	0.0023	0.0079	0.0079	0.0011	0.0011	0.0011						
26	0.0884	0.0884	0.0884	0.0884	0.0884	0.0884	0.0884	0.0884	0.0884	0.0898	0.0884	0.0884	0.0884	0.0884	0.0884	0.0884	0.0634	0.0034	0.0011	0.0068	0.0068	0.0045	0.0045	0.0045	0.0034					
27	0.1148	0.1148	0.1148	0.1148	0.1148	0.1192	0.1163	0.1148	0.1148	0.1177	0.1192	0.1226	0.1148	0.1192	0.1148	0.1148	0.0874	0.1064	0.1097	0.1127	0.1127	0.1125	0.1125	0.1125	0.1111	0.1098				
28	0.1286	0.1286	0.1286	0.1286	0.1286	0.1286	0.1286	0.1286	0.1286	0.1270	0.1286	0.1321	0.1286	0.1286	0.1286	0.1286	0.1118	0.1248	0.1268	0.1298	0.1298	0.1224	0.1224	0.1224	0.1238	0.1270	0.1103			
29	0.1086	0.1086	0.1086	0.1086	0.1086	0.1117	0.1087	0.1086	0.1086	0.1132	0.1117	0.1151	0.1086	0.1117	0.1086	0.1086	0.0711	0.1336	0.1373	0.1342	0.1342	0.1357	0.1357	0.1357	0.1342	0.1375	0.1318	0.1302		
30	0.1146	0.1146	0.1146	0.1146	0.1146	0.1176	0.1147	0.1146	0.1146	0.1191	0.1176	0.1210	0.1146	0.1176	0.1146	0.1146	0.0844	0.1327	0.1363	0.1333	0.1333	0.1348	0.1348	0.1348	0.1333	0.1381	0.1371	0.1431	0.0298	
31	0.1121	0.1121	0.1121	0.1121	0.1121	0.1179	0.1121	0.1121	0.1121	0.1164	0.1179	0.1212	0.1121	0.1179	0.1121	0.1121	0.1001	0.1245	0.1265	0.1294	0.1294	0.1279	0.1279	0.1279	0.1265	0.1266	0.0473	0.1022	0.1164	0.1300

Note :

1) Bangka1, 2) Bangka2, 3) Bangka3, 4) Bangka4, 5) Bangka5, 6) Bangka9, 7) Bangka Selatan1, 8) East Belitung 1, 9) East Belitung 5, 10) East Belitung 6, 11) East Belitung 7, 12) East Belitung 8, 13) East Belitung 9, 14) East Belitung 10, 15) East Belitung 13, 16) East Belitung 12, 17) East Belitung 14, 18) Katingan 5, 19) Katingan 8, 20) Palangkaraya 1, 21) Palangkaraya 2, 22) Palangkaraya 3, 23) Kapuas 1, 24) Kapuas 2, 25) Kapuas3, 26) Kapuas 7, 27) *Osteochilus salsburyi*, 28) *Osteochilus vittatus*, 29) *Squalidus chankaensis*, 30) *Squalidus japonicus*, 31) *Osteochilus waandersi.*

DISCUSSION

The DNA barcode in this research represents the first record using the *CO1* gene on *O. spilurus*. Previous records of this species used the *cytochrome b* gene **(Esa *et al.*, 2012; Kurniawan *et al.*, 2021a)**. The seventeen sequences from the Bangka and Belitung Islands showed low genetic distance. This finding is consistent with the morphological similarity of the species between the two islands, which shows only slight variation **(Kurniawan *et al.*, 2020a)**. Likewise, eight sequences from three different rivers in Kalimantan also showed high similarity. *O. spilurus* from each region can be categorized as an intraspecific clade, as the genetic distance among its members is less than 2% **(Ratnasingham & Hebert, 2013)**. Intraspecific relationships can also occur with genetic distances of up to 3% **(Aminan *et al.*, 2020)**.

Bangka and Belitung, which were separated by the Gaspar Strait around 6,000 years ago **(Voris, 2000)**, do not show significant genetic variation in freshwater fish. The identical environmental conditions on both islands are believed to influence similarities in adaptation and evolutionary direction. These two islands are among the world's largest tin-mining regions. Almost all rivers on the islands are affected by mining activities **(Syarif *et al.*, 2025a)**. Tin mining has the potential to damage fisheries in both Bangka and Belitung **(Ibrahim *et al.*, 2018)**.

The Sebangau, Kapuas, and Katingan Rivers in Kalimantan are associated with blackwater swamp environments **(Thornton *et al.*, 2018; Gandois *et al.*, 2020; Kurniawan *et al.*, 2021b)**. Similar water conditions across these rivers may explain the lack of genetic variation, even though the rivers are not hydrologically connected. Tropical peatlands contribute to extreme chemical and hydrological conditions, producing acidic blackwaters **(Thornton *et al.*, 2019)**.

Intraspecific relationships in Bangka-Belitung and Kalimantan may also be influenced by the absence of geographic barriers such as mountains. Mountains are known to cause geographic isolation, particularly where highlands separate lowland regions within an island **(Machordom and Doadrio, 2001; Wang *et al.*, 2004)**. Mountainous islands generally exhibit greater genetic variation within the same species. For example, Honshu Island in Japan hosts two clades of *Pseudogobio esocinus*, with several sub-clades within them **(Tominaga *et al.*, 2016)**. Java Island, which has many mountains, shows distinct sub-clusters—or even separate clusters—of *Barbodes binotatus* and *Rasbora lateristriata* **(Kusuma *et al.*, 2016; Astuti *et al.*, 2020)**.

The phylogenetic tree based on the *CO1* gene in this study aligns with the findings of **(Kurniawan *et al.*, 2022)**, which separates Bangka-Belitung and Kalimantan into different clades. This separation demonstrates that fish with similar morphological characteristics can exhibit different genetic diversity. Although Bangka-Belitung and Kalimantan were placed into different clades with a genetic distance of 8.7–9.3%, the genetic distance between these clades is still lower than that between *O. spilurus* and other species within the genus *Osteochilus*. Specifically, *O. spilurus* shows genetic distances of 11.5–12.7%

from *O. waandersii*, 12.1–14.7% from *O. microcephalus*, 11.8–13.4% from *O. line*, and 10.9–13.7% from *O. vittatus*. These values support the conclusion that the fish samples in this study do not belong to other species.

Kalimantan and Bangka-Belitung are separated by the Karimata Strait, which was formed approximately 10,000 years ago **(Voris, 2000)**. This long-term geographic isolation is likely a contributing factor to the distinct clades and greater genetic distances observed. Population separation due to oceanic barriers has also been observed in *Rasbora lateristriata* between the islands of Java and Bali. The Bali Strait, formed around the same time as the Karimata Strait, has led to the phylogeographic separation of *R. lateristriata* and *Periophthalmus argentilineatus* **(Kusuma *et al.*, 2016; Arisuryanti, 2018)**. Historical hydrological changes and environmental conditions have resulted in cryptic diversity in several widely distributed species **(Macossay-Cortez *et al.*, 2022)**

CONCLUSION

O. spilurus from Bangka and Belitung Islands shows an intraspecific relationship, as do populations from the Kapuas, Sebangau, and Katingan rivers in Kalimantan. Bangka-Belitung and Kalimantan form distinct clades, which may represent cryptic species, likely due to historical changes such as the formation of the Karimata Strait that separates the two regions. *O. spilurus* has potential to serve as a distribution model for freshwater fish in Sundaland.

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