

# Phylogeography of *Aplocheilus panchax* in Indonesia, with special focus on the Bangka Island population

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**Abstract.** Mustikasari D, Nuryanto A, Suryaningsih S. 2022. Phylogeography of *Aplocheilus panchax* in Indonesia, with special focus on the Bangka Island population. *Biodiversitas* 23: 2035-2046. Previous studies divided Blue panchax, *Aplocheilus panchax* into three different clades, namely West (W), Central (C), and East (E) clades. Blue panchax populations from Indonesia belong to the Central and East clades. However, that study did not include blue panchax samples from pits with harsh conditions in Bangka Island, Indonesia. Therefore, this study aimed to assess the phylogeography of blue panchax in Indonesia with a special focus on the Bangka Island population using the cytochrome c oxidase 1 (COI) gene. The results showed that genetic distance within Bangka Island population was less than 2%, while genetic distances between Bangka population and other populations were ranged from 103.87% to 122.10%. There was also a clear genetic gap between the Bangka and other populations, with the minimum gap was 101.94%. Furthermore, the seventy-nine sequences analyzed resulted 28 haplotypes with genetic variability of 0.221 for nucleotide diversity (p), 0.923 for Haplotype diversity (Hd), 68.028 for Fu's Fs-test, 2.00 ( $P < 0.02$ ) for Fu and Li's D-test, and 2.365 ( $P < 0.02$ ) for Fu and Li's F-test; and 1.927 for Tajima's D test ( $0.10 > P > 0.05$ ). The Bangka population of *Aplocheilus panchax* established a distinct clade from the Western (W), Eastern (E), and Central (C) clades. Molecular data established that the population on Bangka Island is a novel clade for Indonesia and a global blue panchax phylogeographic.

**Keywords:** Abandoned tin mining pits, genetic distance, haplotype, killifish, new clade position

## INTRODUCTION

Phylogeography analyzes and understands organism diversity and biogeography (Bobo-Pinilla et al. 2021). Furthermore, it is a study of geographical distributions of closely related genetic lineages or geographic ordination of genotypes (Rius and Turon 2020). Therefore, phylogeographic research is essential to evaluate the geographical distribution of these genetic lineages and their pattern, as well as elaborate ecology factors and organism biodiversity (Lone et al. 2021).

The blue panchax (*Aplocheilus panchax* Hamilton, 1882) is an endemic species to the Oriental region (Costa 2013; Costa 2016; Beck et al. 2017), widely distributed across the Indo-Malayan Islands, including Indonesia, the Indo-China region, and India (Dekar et al. 2018; Bolotov et al. 2020). *Aplocheilus panchax* is a fish from Genus *Aplocheilus*, Family Aplocheilidae, Suborder Aplocheiloidei, Order Cyprinodontiformes, and Class Actinopterygii (Parenti and Hartel 2011; Furness et al. 2015). Fishes belonging to Order Cyprinodontiformes are also known as *Aplocheiloid killifishes* or *livebearers* (Pohl et al. 2015; Braganca et al. 2018).

Indonesia is one of the world's biodiversity hotspots with many different habitats and a highly complicated geological history (de Bruyn et al. 2014; von Rintelen et al. 2017). Generally, Southeast Asia's complex climatic and geological history caused this region to have high biodiversity (Beck et al. 2017; Fortes et al. 2018).

Biogeography, geology, climate, and ecology of Southeast Asia have led to megadiverse organisms' evolution. Additionally, the region is home to several endemic and ecologically well-adapted species (Hughes 2017; von Rintelen et al. 2017). The *Aplocheilus panchax* can be used to understand further how climatic changes and sea-level fluctuations have influenced the species' distribution within this region. Specifically, sea-level variations result from glacial cycles that continued throughout the Pleistocene, interfering with and restricting the network of all populations. These examples show proof of isolation in palaeodrainage basins (Beck et al. 2017).

Previous phylogeographic study using the COI gene placed the world's *A. panchax* populations into three different clades; West (W), Central (C), and East (E) clades. The *A. panchax* populations from Indonesia were divided into Central and East clades. Central clade consisted of Pekanbaru, Jambi, and West Sumatera populations. East clade was formed by *A. panchax* populations from Java, Bali, Kalimantan, and Sulawesi islands (Beck et al. 2017). Nevertheless, the study by Beck et al. (2017) did not include *A. panchax* samples from Bangka Island. Bangka Island, the biggest tin producer in Indonesia, has unique waters like a lake or pit, known as kolong, formed and abandoned after tin mining activity. This water has low pH (acid waters), low nutrients, minimum dissolved oxygen (DO), and high heavy metals (Ashraf et al. 2011, 2012a, 2012b; Hashim et al. 2018; Koki et al. 2019; Kurniawan 2020). However, *A. panchax*,

locally known as ikan Kepala Timah, can live in this extreme habitat (Kurniawan et al. 2019; Kurniawan et al. 2020; Mustikasari et al. 2020a). The genus *Aplocheilus*, which includes *A. panchax*, was classified as an extremophile fish due to its ability to survive in harsh environmental circumstances (Riesch et al. 2015; Kurniawan and Mustikasari 2021). Mustikasari et al. (2020a, b) explored the presence and morphological variety of blue panchax (*A. panchax*) in the waters contaminated by heavy metals of the abandoned tin mining pits of various ages.

Island biogeography investigates how the richness of the ecosystems and the complexity of the biodiversity may cause speciation. The adaptive radiation, speciation, climate cycles, and topographical complexity can shape island biodiversity (Dorey et al. 2020). Phylogenetic and phylogeographic analyses based on mitochondrial DNA (mt-DNA) are commonly used in island biodiversity exploration. In addition, mt-DNA possesses some characteristics, including cell quantity, genome size, haploid, maternal inheritance, and extremely low probability of paternal leakage, mutation rate, and change mainly caused the mutation. These features make mt-DNA a useful and one of the most often used markers in molecular analysis. The marker has been frequently used to study genetic diversity, population organization, phylogeography, and organism evolution (Gupta et al. 2015).

This study aimed to assess the phylogeography of Blue panchax in Indonesia with a special focus on the Bangka Island population using the cytochrome c oxidase 1 (COI) gene. This is the first time the populations from Bangka Island have been genetically analyzed using the cytochrome oxidase I (COI) gene and compared to other populations on the global distribution of blue panchax. Furthermore, the presence and genetic profile of *A. panchax* can be a model of extremophile fish study. Therefore, it is used as a bioindicator for harsh habitats since the global distribution, ecological and genetic evolution with the Bangka Island population corresponds to others in Indonesian waters.

## MATERIALS AND METHODS

### Study area

The study was conducted in Pangkalpinang City and Bangka District of Bangka Belitung Archipelago Province, Indonesia. Fish samples were collected from abandoned tin mining lakes (pits) of different ages and the Limbung River. Station A and B (< 5 years old), Station C and D (5 - 15 years), Station E and F (15 - 25 years), Station G (25 - 50 years), Station H (50 - 100 years), Station I and J (> 100 years), and Limbung River Stream of Bangka District as Station K such as shown in Figure 1. These ages of research stations indicated the chronosequence of the abandoned tin mining pits were taken place there. There was nothing put in the habitats due to the chronosequence only explain about a succession that happened naturally. In addition, they were related to our previous studies about their characteristics of water quality and the presence of

*Aplocheilus panchax* in the abandoned tin mining pits based on the difference of time (Mustikasari et al. 2020a, b).

### Procedures

#### *Samples preparation for molecular analysis*

The twenty samples were collected at 09.00 am-1.00 pm from closed and open waters of abandoned tin mining lakes (pits) and waters of Limbung River Stream, Bangka Island, using nets with a mesh size of about 0.4 mm. We declared that the collected fish as samples were handled in a good manner, as explained as Bennett et al. (2016), which cite Canadian Council on Animal Care (2005) and American Fisheries Society (2014) about guidelines for the use of fishes in research. They were handled with minimizes pain, distress, suffering and unnecessary loss of external mucus or scales, minimum of the handling duration, to avoid unnecessary stress and exposure time. We also gave attention to life-cycle events, such as aggregations of breeding fish and sensitive habitats were avoided.

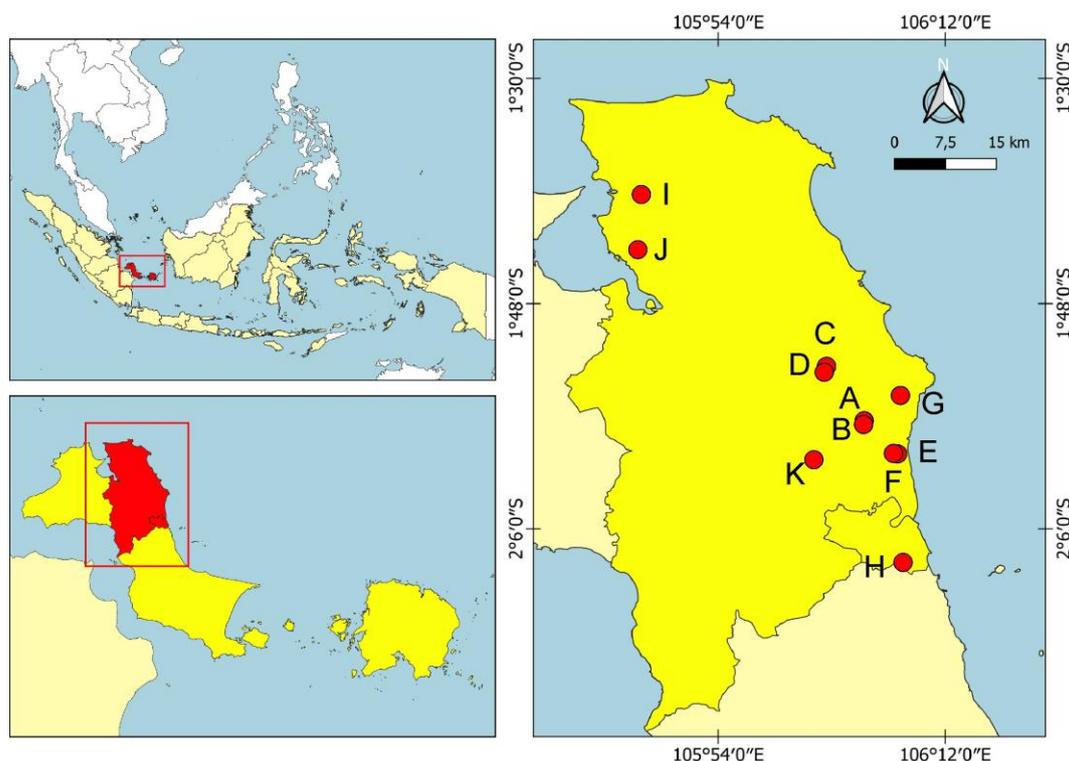
Furthermore, this study utilized a 2.0 ml cryotube with ethanol absolute to preserve each of the fish samples for molecular analysis. The pectoral fin about 2 mm from each dead sample of *A. panchax* was taken for DNA isolation and molecular identification.

#### *Molecular analysis*

The genomic DNA extraction was analyzed with the gSYNC™ DNA Extraction Kit (Geneaid, GS300). Nucleic acid (genomic DNA) concentration was measured using Nanodrop™ 2000/2000c spectrophotometers. Furthermore, molecular analysis was referred to Protocol Species Barcoding Fish GMS-165, Genetika Laboratory of Genetika Science Indonesia, in 2021.

Polymerase chain reaction (PCR) amplification was conducted with (2x) MyTaq HS Red Mix (Bioline, BIO-25048) and KOD FX Neo (Toyobo, KFX-201). The components 1 x 25 µL PCR Master Mix were dd H<sub>2</sub>O 9.5 µL; MyTaq HS Red Mix, 2x 12.5 µL; 10 µM VF2\_t1 0.5 µL; 10 µM Fish F2\_t1 0.5 µL; 10 µM Fish R2\_t1 0.5 µL; 10 µM Fish FR1d\_t1 0.5 µL; and DNA Template 1 µL. Primer sequence of PCR amplification were VF2-t1 5'-TGTA AACGACGCGCCAGTCAACCAACCAAGA CATGGCAC-3'; FR1d-t1 5'-CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA-3'; FishR2\_t1 5' CAGGAAACAGCTATGACACTTCAGGGTGACCGAA GAATCAGAA-3'; and FishF2\_t1 5'-TGTA AACGACGCGCCAGTCTGACTAATCATAAAGATATCGGCAC-3' (Ivanova et al. 2007).

The predenaturation phase initiated the Polymerase Chain Reaction (PCR) cycling for 1 minute (95°C). Subsequently, the actual PCR amplification was conducted for 35 cycles, denaturation process for 15 seconds (95°C), annealing process for 15 minutes (50°C), and extension process for 45 seconds (72°C). The PCR products (1 µL) were assessed by electrophoresis with 1% TBE agarose with Marker 100bp DNA ladder (loaded 2 µL). Furthermore, the quality and length of the PCR products were analyzed by agarose gel electrophoresis. Bi-directional Sequencing conducted the sequencing step at 1st base Asia.



**Figure 1.** Map of research stations at Bangka Island, Indonesia (Mustikasari et al. 2020a, b)

### Data analysis

The contig sequences were obtained from reverse and forward sequences aligned with Program BioEdit (Hall 2011). The phylogenetic study was carried out using MEGA XI (Tamura et al. 2021), utilizing the Maximum Parsimony (MP) statistical approach, and a bootstrap consensus tree was constructed using 1000 replicates. The phylogenetic tree was constructed by comparing sequences of *A. panchax* from Bangka Island with some sequences of *A. panchax* from a previous study (Beck et al. 2017). The study conducted in India (Tamil Nadu and Kolkotta), Cambodia, Vietnam, Thailand (Krabi), Malaysia (Sungai Batu Pahat, Penang, Dungun), Singapore, and Indonesia (Aceh, Pekanbaru, Pulau Laut, West Sumatra, Jambi, Bogor, Surabaya, Banjarmasin, Bali, and Sulawesi) as shown in Figure 2 aim to investigate the position of *A. panchax* population from Bangka Island. Furthermore, it used a sequence of *Aplocheilus andamanicus* (Katwate et al. 2018). The *A. wernerii* (accession number KJ844713.1) was used as outgroup species (Pohl et al. 2015) for this phylogenetic analysis. Sequences metadata from Beck et al. (2017) and Katwate et al. (2018) from NCBI (National Center for Biotechnology Information) were utilized. DnaSP v5 was used to assess the haplotype (Hd) and nucleotide diversity ( $\pi$ ), as well as to perform Fu and Li's F and Tajima's D neutrality tests (Čekovská et al. 2020). A phylogenetic network was constructed according to the median-joining method within the software Network 10. The Kimura 2 Parameter (K2P) genetic distance was calculated in the MEGA XI (Tamura et al. 2021). Genetic distances were utilized to estimate the genetic gap between

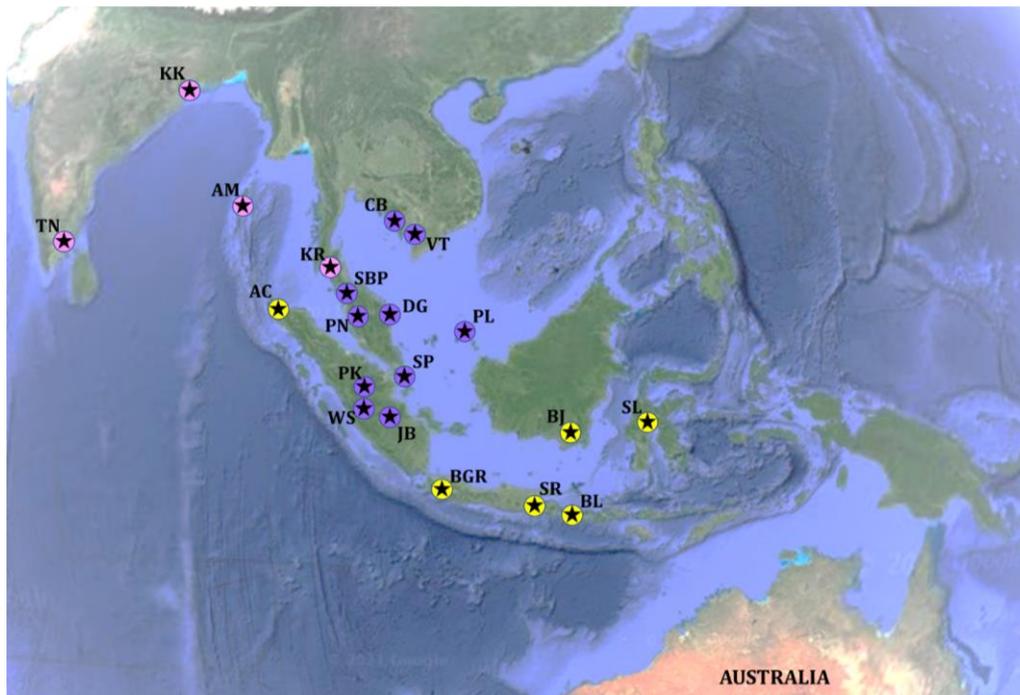
Bangka Island and other clade populations. The lowest genetic distance was estimated by subtracting minimum genetic distance among populations by maximum genetic distance within Bangka Island population.

## RESULTS AND DISCUSSION

### DNA quantity and quality

The successfulness of organism identification methods based on genetic material, such as PCR, relies on the quantity and quality of nucleic acid, purification method, and PCR amplification process. Unfortunately, the low amount and quality of genetic material (DNA) and the appearance of inhibitors inhibit the PCR amplification efficiency (Chowdhury et al. 2016; Dwiyoitno et al. 2018; Kuffel et al. 2021). The analysis of the quantity of sample DNA by Nanodrop spectrophotometer resulted in DNA concentration (ratio A260/280) for all samples between 1.65 and 1.99 (Table 1).

The ratio of absorbance at 260 nm and 280 nm (A260/280) is used to assess the purity of material genetics. The measurement of DNA concentration with Nanodrop was conducted at a wavelength of 260 nm. In comparison, the protein was measured at a wavelength of 280 nm with pure DNA having an absorbance ratio of 260/280 between 1.6 and 2.0 (Setiaputri et al. 2020), 1.7-2.0 (Ruchi et al. 2018), or 1.8-2.0 (Pratomo et al. 2021). The absorbance value below the low absorbance limit indicates the presence of polysaccharides, phenol, and protein contamination.



**Figure 2.** Sampling locations for *Aplocheilus panchax* over 20 areas, namely Tamil Nadu (TN), Kolkotta (KK), Andaman Island (AM) Cambodia (CB), Vietnam (VT), Krabi (KB), Sungai Batu Pahat (SBP), Aceh (AC), Penang (PN), Dungun (DG), Pulau Laut (PL), Singapore (SP), Pekanbaru (PK), West Sumatera (WS), Jambi (JB), Bogor (BG), Surabaya (SR), Banjarmasin (BJ), Bali (BL) and Sulawesi (SL). (Map was reconstructed from Beck et al. 2017 and Katwate et al. 2018)

**Table 1.** Quantity of Sample DNA

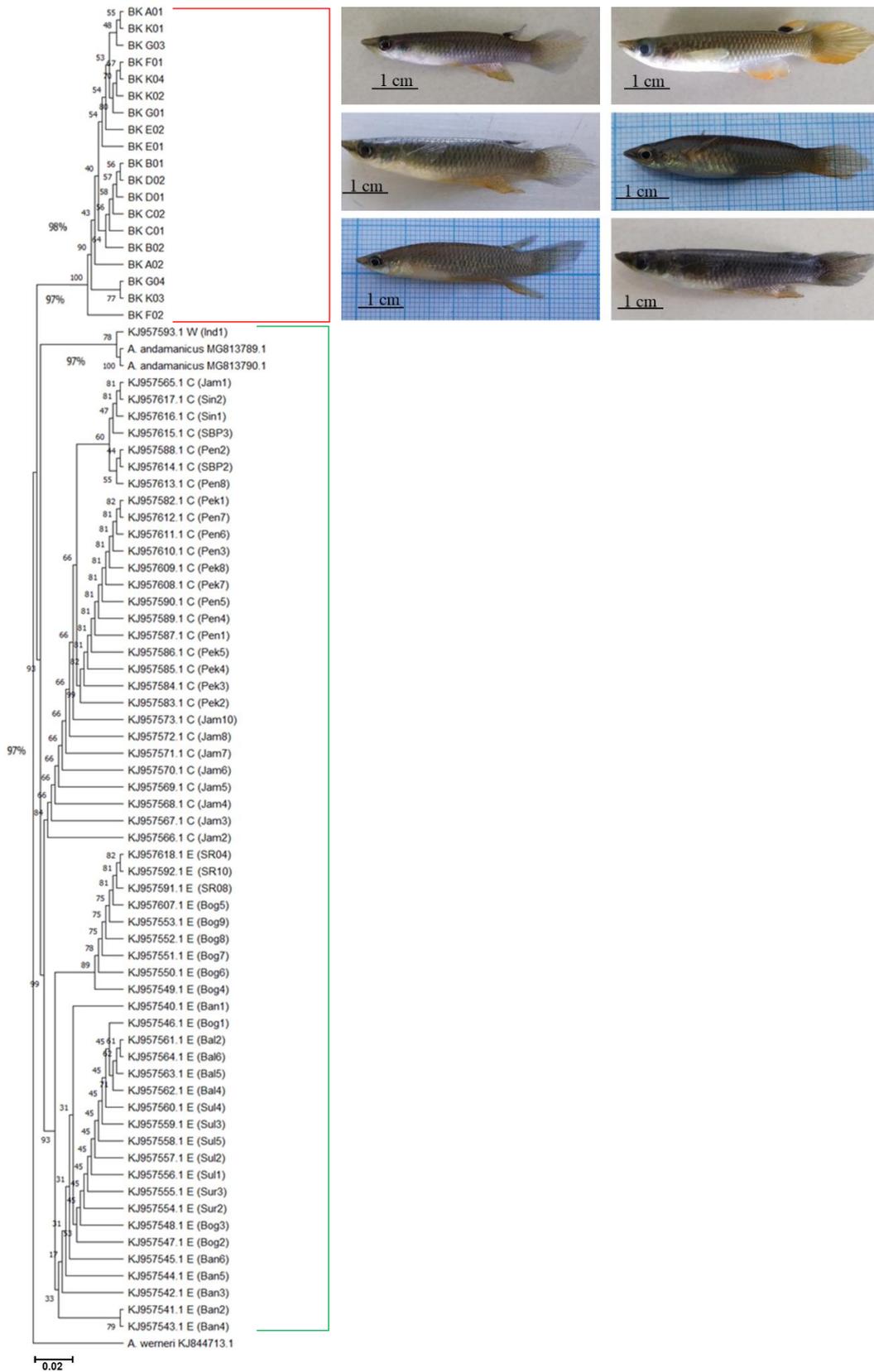
Sample code*	A <sub>260/280</sub>
BK_A01	1.72
BK_A02	1.90
BK_B01	1.91
BK_B02	1.87
BK_C01	1.87
BK_C02	1.91
BK_D01	1.93
BK_D02	1.99
BK_E01	1.90
BK_E02	1.89
BK_F01	1.87
BK_F02	1.65
BK_G01	1.75
BK_G02	1.59
BK_G03	1.81
BK_G04	1.88
BK_K01	1.65
BK_K02	1.83
BK_K03	1.71
BK_K04	1.70

Note: \*) sample' volume (30  $\mu$ L)

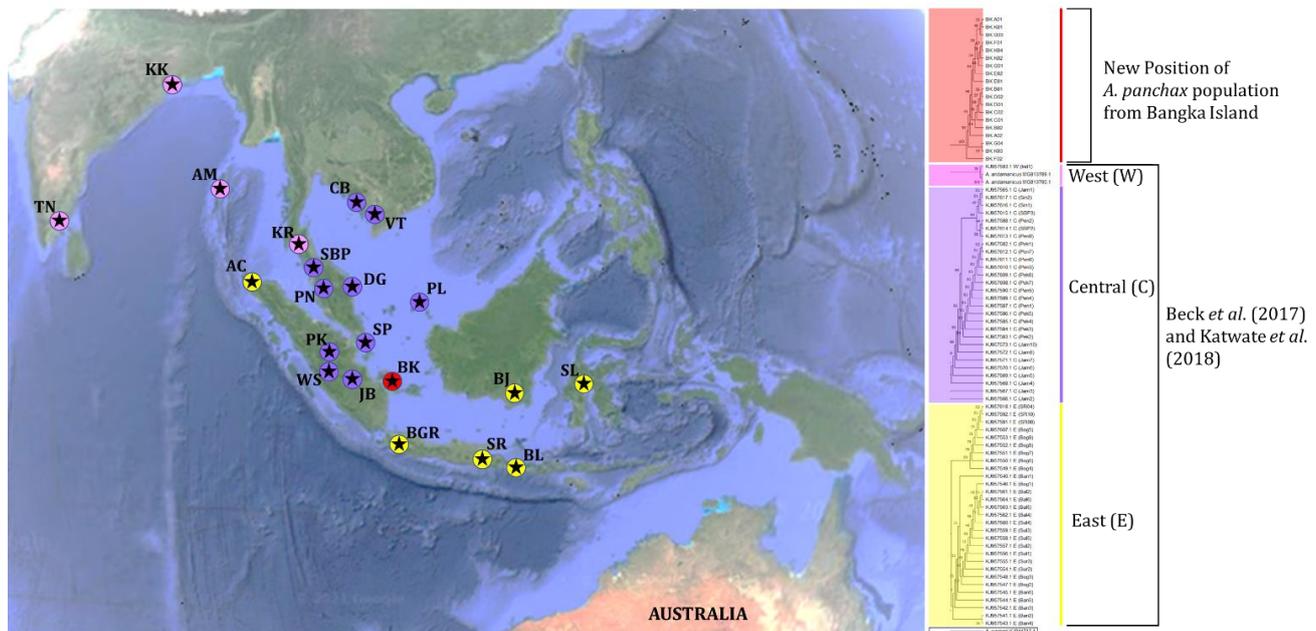
Meanwhile, above the absorbance 2.0 indicates the presence of RNA contamination during the DNA isolation process (Rosilawati et al. 2002; Farmawati et al. 2015; Rizko et al. 2020). The success of a purification extraction

and the quantity of DNA is highly dependent on the isolation of the resulting DNA. Therefore, the isolation process using commercial kits is safer from processing errors that cause contamination. However, studies on several specimens with various treatments stated that not all commercial kits can harvest DNA in high concentrations (Hajibabaei et al. 2006; Setiaputri et al. 2020).

The product of PCR also showed that the COI gene length of *A. panchax* populations from Bangka Island was about 700 bp. Meanwhile, the COI gene length for *A. panchax* was around 621 bp in other studies with accession numbers KJ957593.1, KJ957617.1, and KJ957618.1 from Beck et al. (2017), and also accession number MG813789.1 (Sample *A. andamanicus*) from Katwate et al. (2018). The length showed that the COI gene of *A. panchax* from Bangka Island was the longest. The mitochondrial gene, namely COI, is commonly used for DNA barcoding, about 650 bp and these gene sequences are also used for ecological and evolutionary studies (Yang et al. 2019). The COI gene length differences of *A. panchax* populations indicate a diversity of *A. panchax* around the Oriental region. It plays a vital role in a global study to collect information about biodiversity and be a key in phylogenetic and phylogeographic analyses (Buhay 2009).



**Figure 3.** The phylogenetic position of *Aplocheilus panchax* from Bangka Island is supported by Maximum Parsimony (MP) bootstrap values. Sequences of topotypes of populations from Bangka Island were in red line, while sample's sequences of Beck et al. (2017) and Katwate et al. (2018) which were cited from the existing databases of COI genes, shown in the pink line (West clade), violet line (East clade), and yellow line (East clade), while *Aplocheilus werneri* is outgroup.



**Figure 4.** New phylogeographical map for *Aplocheilus panchax*, particularly Bangka Island populations (red circle) on global phylogeographic of blue panchax

#### The phylogenetic tree and phylogeographic of *A. panchax* population from Bangka Island

The phylogenetic tree indicated that the ancestral relationship between the *A. panchax* from all over their geographic distribution based on the COI gene sequences was well supported by high bootstrap value (>90% bootstrap values). The phylogenetic tree analysis showed *A. panchax* widespread throughout the Oriental region, including Indonesia as a part of Southeast Asia countries. However, the population from Bangka Island has different COI sequences and is separated from others. Bangka Island individuals formed a distinct clade from those previously described by Beck et al. (2017) and Katwate et al. (2018). (Figure 3).

Beck et al. (2017) revealed their research about Bayesian posterior probabilities that displayed for Western (W) clade, Eastern (E) clade, and Central (C) clade of *A. panchax*. We added a novel clade to previous phylogenetic tree (Beck et al. 2017; Katwate et al. 2018) by adding samples from Bangka Island (Figure 3). Therefore, the authors also recommended a new map reconstruction of the global phylogeographic on blue panchax (*A. panchax*) gene distribution (Figure 4).

A previous study by Katwate et al. (2018) denied the presence of *A. panchax* in the Indo-Malay region. They provided morphological and molecular evidence and demonstrated that *A. andamanicus* and *A. armatus* distinct and valid killifish species in the Indo-Malay region. However, the present study supported Beck et al. (2017) by showing that the killifish from Indonesia, especially populations from Bangka Island, is *A. panchax*. The present result was supported by morphological data that killifish from Bangka Island is *A. panchax* (Mustikasari et al. 2020b).

Several factors can impact an individual's genetic diversity and population genetic structure, such as climate or environmental change, natural boundaries, environmental variables, movement and migration, and human activities (Nater et al. 2013; Wang et al. 2020). For example, the persistence of climate change is determined by life history characters of organisms, such as dispersal ability, generation period, reproductive ability, habitat specialization, organism interactions, genetic diversity, and habitat or migration corridors (Schierenbeck 2017).

The critical part was that the position of *A. panchax* populations from Bangka Island was different from other locations, but they were included in Sundaland region. It indicated that the biogeographic region of Sundaland (Borneo, Sumatra, the Malay Peninsula, Java, Palawan, and associated islands) is essential to understand evolution (Hinckley et al. 2022). As long as chronosequence exists, the ecological component may affect organism diversity. Ecological disturbance is necessary to maintain the dynamism and diversity of ecosystems. The disturbance history may be the primary driver that shapes patterns of genetic diversity in many natural populations through changes (Banks et al. 2013). Beck et al. (2017) explained that the significant mitochondrial clades of *A. Panchax* are consistent with this study. The basal dissimilarity of *A. panchax* mitochondrial ancestries was around 3.5 million years ago (Ma).

On the other hand, the subsequent dissimilarity timings of these clades occurred in the early Pleistocene (~2.6 Ma). Ceaseless phylogeographic investigation showed a reasonable west-east dispersal followed by quick radiation across Southeast Asia. Salles et al. (2021) recreate scene evolution, sedimentation, and Sundaland flooding history under tectonic, eustatic, and precipitation constraining

conditions. Furthermore, they link Sundaland's flooding history to tectonic and sea-level conditions using three external driving instruments of rainfalls, eustatic sea-level variations, and tectonics. Over the last one million years, these factors caused the Mekong, Johor, Siam, and East Sunda River to merge. Additionally, it caused Bangka Island to be separated from the mainland of Sumatra Island, Malay Peninsula, Java, and Kalimantan, about 400 thousand years ago (ka).

Founder effects or events can impact stochasticity in a species' genetic population structure at the regional scale (Haileselassie et al. 2018). Even though their populations have decreased in genetic diversity after the founder event, specific individuals that establish well in new places appear to be destined to extinction. The limit of life forms to adjust to new environments can rely upon the organism's capacity of reacting to regular determination, which is dictated by the genetic pattern of the founder populations (Lee 2002; Dlugosch and Parker 2008; Kaňuch et al. 2014). This condition can impact genetic variation in natural populations, mutation, and genetic drift (Star and Spencer 2013). Genetic drift and gene flow shape allele frequencies over time for an extended period (Chen et al. 2019). Ecological and geological factors may have contributed to the high degree of divergence in COI gene between *A. panchax* populations from Bangka Island and other sites. Subsequently, *A. panchax* sequences from all clades were also analyzed, with a genetic distance of 0.00% to 1.93% for within Bangka Island population. Genetic distance between Bangka Island and other clade populations ranged from 103.87% to 122.10%. Maximum intrapopulation genetic distances within Bangka Island, East clade, and Central clade populations were less than 2%, while within West clade populations had a maximum genetic distance larger than 2% (Table 2). As a result, there was a clear genetic gap between the Bangka Island population and other populations, with a minimum gap 101.94% (Bangka Island and Central Clade populations) and a maximum gap value was 108.52% (Bangka Island and West clade populations). These values made Bangka Island populations were significantly different and separated from other clade populations (Figure 5).

The genetic distances were lower than 2% (0.02), indicating that *A. panchax* populations from Bangka Island did not indicate cryptic species. However, the phylogenetics showed that *A. panchax* populations from

Bangka Island differed from others. Intraspecific genetic distances in some species are higher than 2% (Thu et al. 2019) or above 3% (Nascimento et al. 2016), indicating the existence of cryptic diversity within these fishes. The term "cryptic species" has lately replaced the term "siblings" for taxa of this type (Korshunova et al. 2019). Sibling refers to two or more distinct individuals classified as a single species under one or the same scientific name (Bickford et al. 2007; Xiao et al. 2010; Karanovic et al. 2016). These species cannot be confidently separated based on their morphology, yet they were genetically distinct (Boluda et al. 2016; De Oliveira et al. 2017; Faulwetter et al. 2017). The cryptic species were delineated as individuals in the same geographic area but exhibited significant molecular phylogenetic contrasts. However, they are not recognized morphologically and ethologically (Hosoishi and Ogata 2019; Cerca et al. 2020).

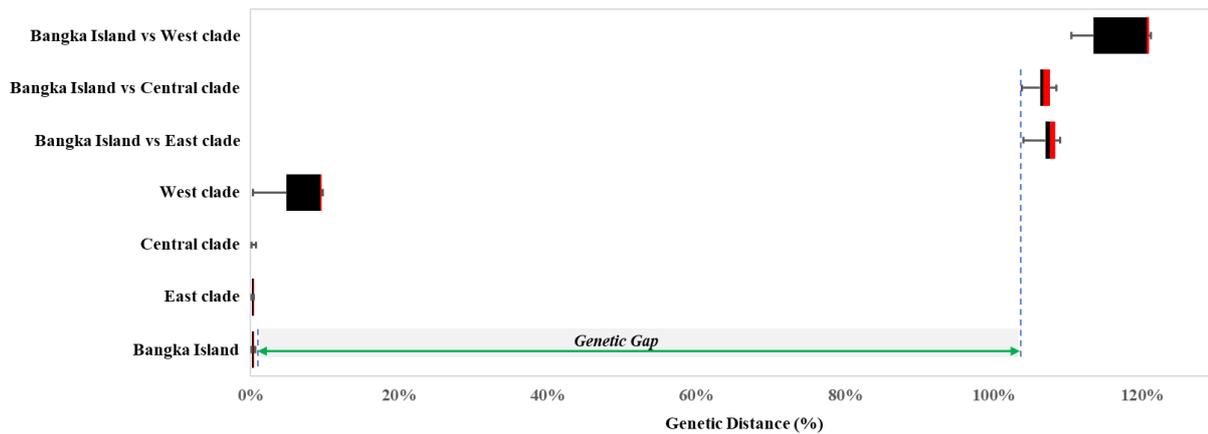
The seventy-nine (79) sequences that were analyzed by DNAsp v.5 showed genetic variability of 0.22 for nucleotide diversity (p), 0.895 for Haplotype diversity (Hd), 68.028 for Fu's Fs test, 2.00 ( $P < 0.02$ ) for Fu and Li's D' test, and 2.365 ( $P < 0.02$ ) for Fu and Li's test. There were 28 haplotypes of these specimens among the COI sequences (n: 79) built into a haplotype network (Figure 6). All of the sequences analyzed showed that the haplotype network has a star-shaped topology.

Hap\_1 to Hap\_15 showed haplotype of *A. panchax* populations from Bangka Island, while Hap\_16 to Hap\_28 were from Beck et al. (2017) and Katwate et al. (2018). Current approaches to biodiversity research focus primarily on ecosystems, environmental communities, geographic regions, and species (Coates et al. 2018). Ecological factors such as over-exploitation, pollution, habitat destruction, and climate change can substantially impact intra-population genetic variation (Liu et al. 2013; Martinez et al. 2018). Recent studies have shown a strong linkage between environmental pressures and biodiversity levels, including genes, species, populations, and communities. The relationships between genetic diversity, polymorphism, and ecological stress have been evidenced in natural populations. The biochemical and molecular approaches investigated the correlation between the genetic structure of populations and the environmental characteristics (Cimmaruta et al. 2003; Markert et al. 2010; Schierenbeck 2017; Hu et al. 2020).

**Table 2.** The genetic distances within and among clades of *Aplocheilus*

<i>Aplocheilus panchax</i> Populations	Genetic Distance (%)			
	[1]	[2]	[3]	[4]
Bangka Island [1]	0.00 - 1.93			
East Clade [2]	104.02-110.32	0.00 - 1.76		
Central Clade [3]	103.87 - 108.49	1.40 - 2.48	0.00 - 1.05	
West Clade [4]	110.45 - 112.10	7.37 - 12.54	6.98 - 13.22	0.00* - 9.80**

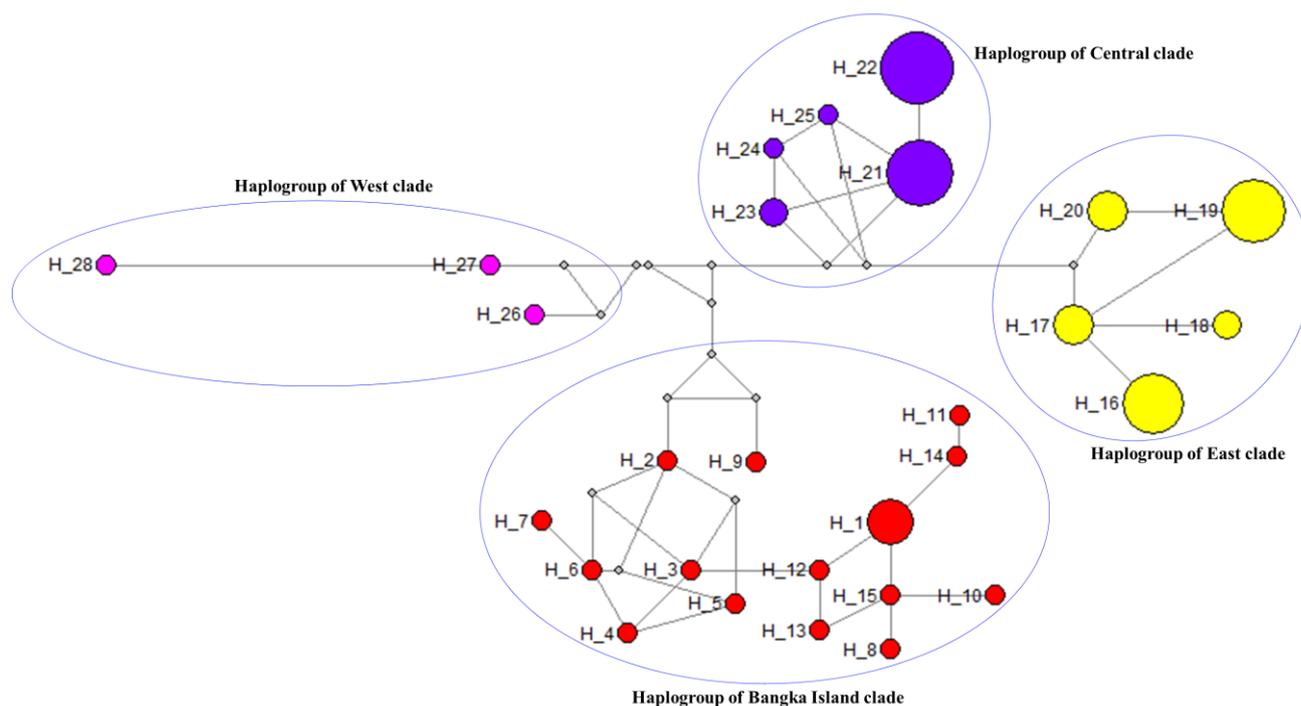
Note: Values in bold were intra-population genetic distances of *Aplocheilus*, \* there was indication a differences *A. panchax* and *A. andamanicus* as well as Katwate et al. (2018)



**Figure 5.** Intra- and inter-species genetic distances for the *Aplocheilus panchax* populations from Bangka Island and other clades. There was a clear genetic gap, spanning from 1.42% to 103.87%, between the maximum within Bangka Island populations and minimum inter populations distance that indicates the *Aplocheilus panchax* populations from Bangka Island were genetically distinct from each other clade. Black lines within the boxes showed the medians, and the red boxes indicated the 75<sup>th</sup> quartiles

**Table 3.** The haplotype of *Aplocheilus panchax* on each clade

Haplotype(s)	Specimen(s)
Hap_1: 5	[BK_A01 BK_A02 BK_E01 BK_E02 BK_G03]
Hap_2: 1	[BK_B01]
Hap_3: 1	[BK_B02]
Hap_4: 1	[BK_C01]
Hap_5: 1	[BK_C02]
Hap_6: 1	[BK_D01]
Hap_7: 1	[BK_D02]
Hap_8: 1	[BK_F01]
Hap_9: 1	[BK_F02]
Hap_10: 1	[BK_G01]
Hap_11: 1	[BK_G04]
Hap_12: 1	[BK_K01]
Hap_13: 1	[BK_K02]
Hap_14: 1	[BK_K03]
Hap_15: 1	[BK_K04]
Hap_16: 9	[KJ957618.1 KJ957549.1 KJ957550.1 KJ957551.1 KJ957552.1 KJ957553.1 KJ957607.1 KJ957591.1 KJ957592.1]
Hap_17: 4	[KJ957540.1 KJ957542.1 KJ957544.1 KJ957545.1]
Hap_18: 2	[KJ957541.1 KJ957543.1]
Hap_19: 10	[KJ957546.1 KJ957547.1 KJ957548.1 KJ957554.1 KJ957555.1 KJ957556.1 KJ957557.1 KJ957558.1 KJ957559.1 KJ957560.1]
Hap_20: 4	[KJ957561.1 KJ957562.1 KJ957563.1 KJ957564.1]
Hap_21: 11	[KJ957565.1 KJ957566.1 KJ957567.1 KJ957568.1 KJ957569.1 KJ957570.1 KJ957571.1 KJ957572.1 KJ957573.1 KJ957616.1 KJ957617.1]
Hap_22: 13	[KJ957582.1 KJ957583.1 KJ957584.1 KJ957585.1 KJ957586.1 KJ957587.1 KJ957589.1 KJ957590.1 KJ957608.1 KJ957609.1 KJ957610.1 KJ957611.1 KJ957612.1]
Hap_23: 2	[KJ957588.1 KJ957613.1]
Hap_24: 1	[KJ957614.1]
Hap_25: 1	[KJ957615.1]
Hap_26: 1	[KJ957593.1]
Hap_27: 1	[ <i>A. andamanicus</i> MH813789.1]
Hap_28: 1	[ <i>A. andamanicus</i> MH813790.1]



**Figure 6.** Median-joining network based on COI sequences indicating the *Aplocheilus panchax* populations of Bangka Island (red haplogroup) formed distinct clade from other clades from Beck et al. (2017) and Katwate et al. (2018). Numbers correspond to haplotype. Crossing-line indicated mutated positions. Different colors showed the collection sites: red (Bangka Island clade), yellow (East clade), violet (Central clade), and pink (West clade), while grey (median vectors) and blue circle (haplogroup). Colored-circles size was proportional to haplotype frequency (Table 3)

The water characteristic of tin mining pits as one of the habitats for *A. panchax* has an acidic pH and high heavy metals contamination (Kurniawan et al. 2019; Kurniawan 2020). A recent study proved that the waters in abandoned tin mining pits are contaminated by heavy metals such as As, Co, Cr, Cu, Fe, Ga, Hf, Mn, Ni, Pb, Sn, Ta, Te, Th, V, and Zn. Heavy metal presence corresponded with pH characteristics (Kurniawan 2020). Therefore, the pH value, acidic pH, specifically acidic mine drainage (AMD) due to the oxidation process of sulfide minerals and potentially acidic formation (PAF), is a significant indicator of abandoned post-mining habitats (Tan et al. 2007; Çelebi and Öncel 2016). These conditions cause organisms to adapt to the extreme environment since *A. panchax* was grouped as extremophile fishes (Riesch et al. 2015; Kurniawan and Mustikasari 2021). The environmental factor from Bangka Island as a tin producer may also contribute to the genetic diversity of *A. panchax*.

Previous studies investigated the correlation between the water quality of abandoned tin mining waters with the presence and morphological characteristics of *A. panchax*. The results showed that *A. panchax* was found in pits with a pH value of 3.81-3.84 and dissolved oxygen (DO) between 5.33 and 5.63. The change of chronosequence's pH impacted the other changes such as DO, BOD, C-organic, total nitrogen, total phosphate, and others (Kurniawan et al. 2019). The presences and phenotypic characters were correlated with the environmental factors, especially pH and heavy metals (Mustikasari et al. 2020a,

b). Therefore, these factors strongly contributed to the polymorphism of *A. panchax* populations from Bangka Island.

Genetic diversity is considered an internal contributing element in the susceptibility of organisms to heavy metals-related poison or toxicity levels. The variety in various genes, directly or indirectly included in the metabolism of weighty metals, has been researched by specific studies. For example, metallothioneins (MTs) are proteins that detoxify heavy metals because of a few gene varieties of genomic sequences (Joneidi et al. 2019). Metallothioneins are small cysteine-rich proteins that play significant roles in metal homeostasis and protection for heavy metal toxicity, DNA defect, and oxidative conditions (Si and Lang 2018), cellular processes, cell growth regulation, and well as proliferation and DNA repair (Grennan 2011).

The contribution of MTs in various cell or organelles processes has gotten much consideration, while their association with the mitochondria functions has been inadequate. Furthermore, it increases the duration of malfunctioning mitochondrial cells by protecting productive components from the damage caused by reactive oxygen species (ROS) and limiting apoptosis. MTs are also involved in mitochondrial infection, including redox balance, metal homeostasis, enzyme, and transcription factor regulation (Lindeque et al. 2010; Kurniawan and Mustikasari 2021). The requirements for obtaining metal specificity and specific novel capacity may drive their enhancement. MTs further enhanced the

capability of metal detoxification under ecologically sensitive settings (Nam and Kim 2017). The relationship with mitochondria indicated an extreme environment as in abandoned tin mining waters of Bangka Island to genetic diversity, especially the COI gene of *A. panchax*. The heavy metals contamination and acidic pH in the habitat can cause genetic variations in mitochondrial genes, such as the COI gene. Moreover, heavy metals can reduce genetic variability within natural populations and cause genetic erosion (Ungherese et al. 2010). The evolution chronosequence of Bangka Island and the entire Sundaland may be attributed to the divergence of COI gene changes to diversify *A. panchax* genes.

It could be concluded that *Aplocheilus panchax* from the Bangka Island was highly divergent from other populations, including Indonesian populations with a high genetic gap. The *A. panchax* population on Bangka Island formed a novel clade for Indonesia and in a global blue panchax phylogeographic.

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