

Phylogeography of *Anabas testudineus* Bloch, 1792 (Pisces: Anabantidae) in Asia

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Abstract. *Anabas testudineus*, the climbing perch, is a valuable and well-liked freshwater fish with significant commercial worth. This fish is found in Indonesia and other parts of Asia. Nonetheless, there hasn't been much research done on the genetic relationships across Asian populations of climbing perch. Thus, the aim of this work was to use the mitochondrial COI gene to examine the phylogeography of *A. testudineus* in freshwater Asia. The population sample was taken from South Aceh, Indonesia. The sample was extracted using a modified C-TAB protocol. In all, 48 sequences from the Genbank and three sequences from South Aceh were included in this investigation. Seven samples came from Indonesia, three from Malaysia, nine from Philippines, seven from Vietnam, eight from Thailand, ten from India, and four from Bangladesh. According to the findings, the sequences formed 17 different haplotypes. For every population, the haplotype diversity (Hd) value is 0.897. The genetic distance measuring the closest populations is 0.0007 between Indonesia and Philippines, whereas Indonesia and Vietnam populations are the furthest apart, at 0.0904. Between 0 and 0.0157 is the genetic distance within the population. Thus, the populations of *A. testudineus* in Aceh and Indonesia and the Philippines are genetically more similar.

1 Introduction

The climbing perch, *Anabas testudineus*, are economically significant in Indonesia [1]. *A. testudineus* are typically found in rice fields, swamp, and ditches and ponds that have overflowing water or are connected to open water channels [2, 3]. There is a tendency for *A.*

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testudineus populations to decline in their natural habitats, and this is assumed to be a result of a number of stresses, including intensive fishing and alterations in the environment [4-6].

This work analyzed the phylogeographic pattern of *A. testudineus* from Asia using the genomic technique and the COI gene marker. Combining phylogenetics and geography, phylogeography analysis studies how lineages of a species are distributed geographically [7]. Prior research on freshwater fish phylogeographic pattern comparisons in significant basins in the trans-Andean area and Lower Central America has been published [8], Lake Kolleru, Andhra Pradesh, India [9], genetic diversity and phylogenetic relationship among Anabantoidei fish in South Kalimantan [10], the Indonesian phylogeography of *Aplocheilichthys panchax* [7], and the Arabian Sea's Bengal Strait's phylogeographic pattern snapper [11].

For the purpose of creating a better management strategy, it is crucial to comprehend how biogeography and species' life histories relate to one another through the study of phylogeography. Fish that are genetically distant from one another need to be maintained differently from species that are closely related to one another. As a result, the current study's objective was to examine the phylogeography of *A. testudineus* across Asia in order to provide crucial data for creating an improved management plan for this species in Indonesia.

2 Material and Methods

2.1 Collection of tissue sample

In this work, two different kinds of sequencing data were used: primary and secondary data. The samples of *A. testudineus* populations from Aceh (as primary data), As secondary data from the NCBI Genbank, populations from Indonesia, Malaysia, the Philippines, Vietnam, Thailand, India, and Bangladesh were used to represent samples from Asia (Figure 1).

Three samples of the Aceh population's *A. testudineus* were taken from the Singkil peat swamp in South Aceh, Aceh Province on July 2023, Indonesia. After the sample was taken for documentation and around 1 centimeter of the pectoral fins were removed with sterile scissors, it was preserved in a 96% ethanol solution. Fish tissues are documented and collected using the protocol created by the Fish-BOL cooperation [12]. The samples were brought to the lab so that they could be examined further. For Comparative Phylogeography study, secondary data on *A. testudineus* sequences from Bangladesh, Vietnam, Thailand, India, Malaysia, Indonesia, and the Philippines were obtained from The GenBank. The sources of the sample sources are displayed in Figure 1 and Table 1.

2.2 DNA isolation

Modified Cetyltrimethyl Ammonium Bromide (CTAB) was used to extract DNA [13, 14]. In a 1.5 mL sterile tube, chopped fin tissue was added, which 700 microliters of Cetyltrimethyl Ammonium Bromide (CTAB) and 3 microliters of Proteinase K were added, after which it was vortexed for 15 seconds and incubated for 3 hours at 60°C. Following incubation, 700 µl of Chloroform Isoamyl Alcohol (CIA) were added, and after 30 seconds of revortexes, the mixture was centrifuged for 15 minutes at 11000 rpm. After transferring the supernatant to a new 1.5 mL tube and adding 100% ethanol, the tube was vortexed for 30 seconds again and centrifuged for 15 minutes at 12000 rpm. After discarding the produced supernatant and rinsing the tube with 70% ethanol, the tube was tapped on a dry tissue to remove any remaining ethanol, and it was allowed to dry for ten minutes at room temperature. Subsequently, the DNA sample was added 60 µl of DDH₂O was added. It was then stored at -20°C for future use.

2.3 Amplification of DNA

Amplify up samples of the extraction product, Polymerase Chain Reaction (PCR) was utilized in vitro. using both forward and reverse primers: Fish F1 - 5' TCA ACC AAC CAC AAA GAC ATT GGC AC-3', and Fish R1 - 5' TAG ACT TCT GGG TGG CCA AAG AAT CA-3' [15]. 25 μ l of DNA template, 8.5 μ l of ddH₂O, 1 μ l of forward primer, 1 μ l of reverse primer, and 12.5 μ l of red master mix were used in the PCR. A PCR equipment (Senso Quest Lab cyler) with 30 cycles was used to perform the PCR procedure. The steps in each cycle are as follows: two minutes at 95 °C for pre-denaturation, 45 seconds at 94 °C for denaturation, 45 seconds at 49.7 °C for annealing, 60 seconds at 72 °C for extension, and 10 minutes at 72 °C for final extension before cooling at 4 °C [16]. Two microliters of the PCR product were taken, placed in agarose wells, and electrophoresed in 2% agarose for thirty minutes at 100 volts. Using UVITEC FIRERIDER V10, DNA was seen. Selected for sequencing at First Base Laboratories in Malaysia are the clear and bright DNA bands.

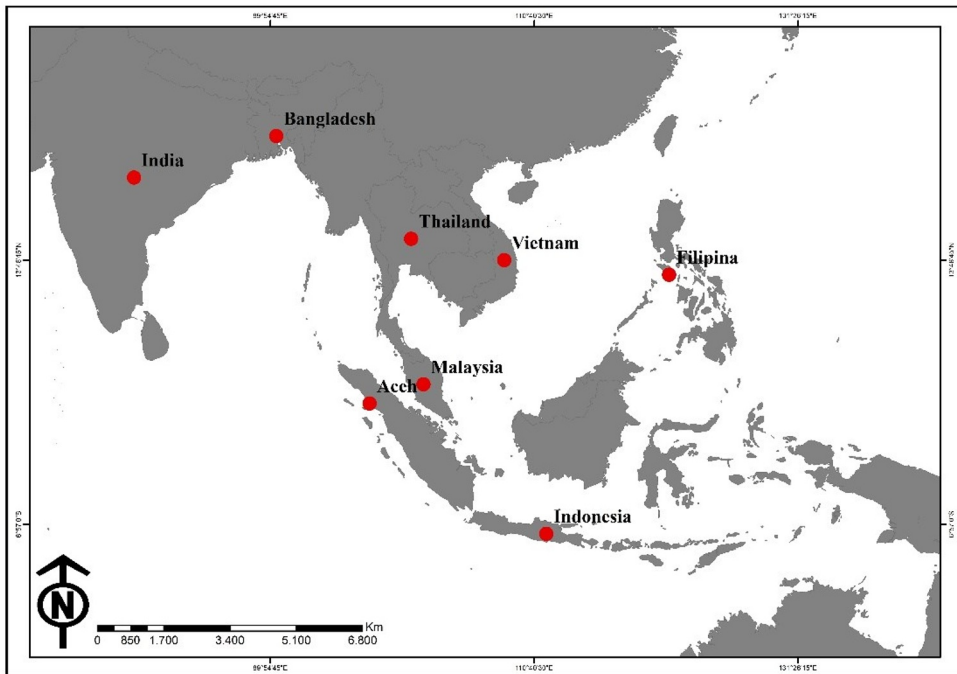


Fig. 1. The research map (the red dot signifying the *A. testudineus* population in Asia)

2.4 Data analysis of mitochondrial DNA

MEGA 11 software was used to analyze and modify the sequencing data in order to verify the taxonomic status. The data from the Basic Local Alignment Search Tool (BLAST) was then compared with the sequences [17]. Using MEGA 11, the genetic distance was determined using the Kimura-2 parameter (K2P) model. The phylogenetic tree was created using the Neighbour Joining (NJ) method with a 10.000x bootstrap and the Kimura-2 parameter (K2P) model. Additionally, the distribution of haplotypes was examined using DNASP 5.10 [18]. While DNA polymorphism, haplotype diversity (Hd), and nucleotide diversity (π) were examined using ARLEQUIN software.

Table 1. *Anabas testudineus* sample IDs for main and secondary data from eight Asian populations.

No.	Country/ population	Total Sample	Total Haplotype	Sample ID
1	Aceh	3	2	SM 12, SM 13, SM 14
2	Indonesia	7	4	KU692244, KU692243, KM213039, KM213038, KU692242, KU692240, KU692241
3	Malaysia	3	2	MW590991, MW590990, JF781185
4	Philippines	9	1	MG407353, MG407352, MG407351, MG407350, MG407349, JN021211, HQ682666, HQ682665, HQ682664
5	Vietnam	7	4	MK368521, MK368520, MK368519, MK351908, KF752456, KF752454, KF752455
6	Thailand	8	4	MK628410, MK049492, MK049491, JQ661371, MK448172, MK448171, JQ661370, JQ661369
7	India	10	3	JX260823, JX260824, MK213551, MK213550, MK213552, JX983213, JX983214, KY356781, KY356780, KY356761
8	Bangladesh	4	2	MK572026, KX455903, MN083164, MN083163

3 Results and Discussion

Seventeen haplotypes were obtained from a total of 51 sequences derived from 8 populations, with an average fragment length of 608 bp. Seven population samples from Indonesia yielded four haplotypes; three samples from Malaysia yielded two haplotypes; one haplotype was from the Philippines population; Vietnam yielded four haplotypes; Thailand yielded four haplotypes; India generated three haplotypes; and Bangladesh supplied two haplotypes, all in all. In the meanwhile, 2 haplotypes were generated from 3 samples in the Aceh population. All populations had nucleotide diversity ranging from 0 to 0.04 and haplotype diversity ranging from 0 to 0.3. The populations in Aceh and Malaysia had the most diversity (value > 0.3), while the population in the Philippines had the lowest diversity (value = 0). Table 2 displays the results for nucleotide diversity (π), total haplotype (Hn), and haplotype diversity (Hd). The Malaysian population had the greatest genetic distance (interspecific) within populations (0.016), while the populations in the Philippines had the lowest (value of 0).

Table 3 shows that the Indonesian population with Vietnam had the greatest intraspecific genetic distance (0.090). *A. testudineus* is descended from a single ancestor, according to phylogenetic connections analysis, with all samples belonging to one major clade and three sister clades (Figure 3). Filipina, Aceh, dan Indonesia termasuk dalam sister clade 1 yang sama. India dan Bangladesh termasuk dalam sister clade lainnya, sedangkan Vietnam, Malaysia, dan Thailand termasuk dalam sister clade 2. Figures 2 and 4 depict the connectivity population, with populations in Malaysia and Thailand sharing haplotype No. 1. Populations in Indonesia and the Philippines share Haplotype No. 3. While the other haplotypes are

independent, the populations of Bangladesh and India share haplotype No. 16 and Indonesia and Aceh share haplotype No. 4.

Table 2. Analysis of *A. testudineus* genetic diversity among eight Asian populations. Three measures of haplotype diversity (*Hd*), total haplotype (*Hn*), and nucleotide diversity (π)

Species	Population	N	Genetic Diversity		
			<i>Hn</i>	<i>Hd</i>	π
<i>Anabas testudineus</i>	Aceh	3	2	0.314	0.002
	Indonesia	7	4	0.181	0.001
	Malaysia	3	2	0.314	0.039
	Philippines	9	1	0	0
	Vietnam	7	4	0.181	0.002
	Thailand	8	4	0.139	0.002
	India	10	3	0.104	0.001
	Bangladesh	4	2	0.204	0.001

The population of Aceh and Malaysia had the most haplotype diversity (value = 0.314), while the population of the Philippines had the lowest haplotype diversity (value = 0), according to the research. Based on [19] the haplotype diversity frequency is divided into three categories: low (0.1–0.4), moderate (0.5–0.7), and high (0.8–1.00). Consequently, the total haplotype diversity of the Asian *A. testudineus* population falls into the low range. Furthermore, Hendiari, Sartimbul, Arthana and Kartika [20] claimed that currents and geographic closeness are the main causes of high genetic diversity, whereas habitat conditions and overexploitation are the main causes of low genetic variety in a population [21].

Table 3. The genetic distance between eight populations of *A. testudineus* using the COI gene, as well as the genetic distance within each group (bold).

No	Location	1	2	3	4	5	6	7	8
1	Aceh	0.002							
2	Bangladesh	0.084	0.001						
3	India	0.084	0.002	0.001					
4	Indonesia	0.002	0.083	0.083	0.001				
5	Malaysia	0.087	0.081	0.083	0.087	0.016			
6	Philippines	0.002	0.082	0.082	0.001	0.086	0		
7	Thailand	0.086	0.078	0.080	0.085	0.009	0.084	0.002	
8	Vietnam	0.088	0.085	0.087	0.090	0.018	0.090	0.025	0.002

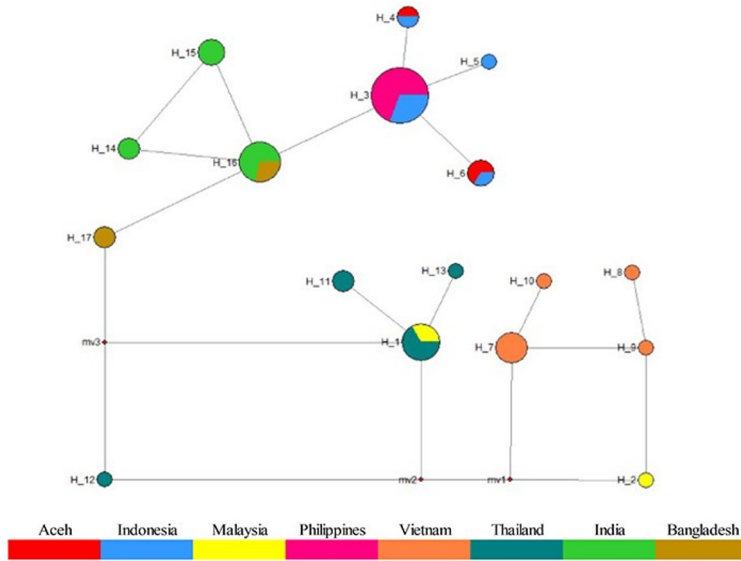


Fig. 2. The haplotype networks of *A. testudineus* from eight Asian populations

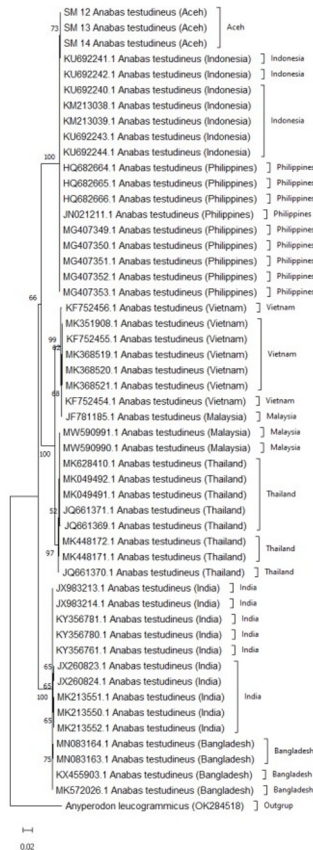


Fig. 3. The phylogenetic tree of 51 *A. testudineus* individuals from eight Asian populations

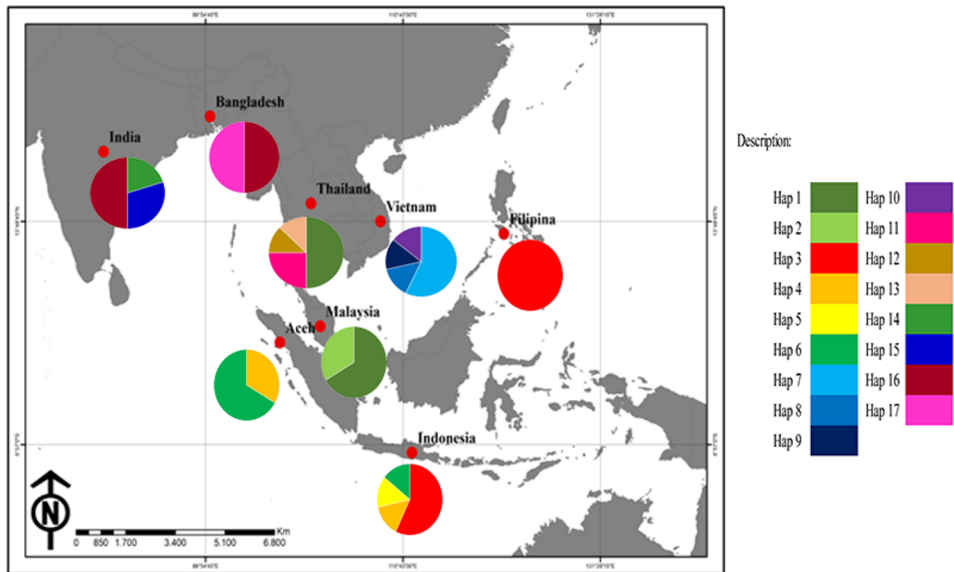


Fig. 4. The distribution map of *A. testudineus* haplotypes from eight Asian populations

One primary clade that was split into three sister clades is produced by reconstructing the phylogenetic tree using 51 sequences from eight populations: Aceh, Indonesia, Malaysia, Philippines, Vietnam, Thailand, India, and Bangladesh. This suggests that the progenitor was the source of the tight relationship between these groups. The genetic separation between clades provided support for this phylogenetic tree. [19]. The Malaysian population had the maximum genetic distance (interspecific) within populations (0.016), whereas the populations in the Philippines had the lowest (0). According to Pilot, Dahlheim and Hoelzel [20], populations with lower genetic distance values have tighter family ties, whereas populations with greater genetic distance values have farther-off kinship ties.

Populations in Asia are linked by shared haplotypes, as evidenced by the connectivity analysis between populations. For example, haplotype No. 1 was found in populations in Malaysia and Thailand, haplotype No. 3 in populations in Indonesia and the Philippines, haplotype No. 4 in populations in Indonesia and Aceh, and haplotype No. 16 in two populations, India and Bangladesh. The same geographic location is the cause of population connection, according to Saleky and Dailami [21]. Compared to other Asian nations, Aceh and Malaysia have greater levels of genetic variation in their *A. testudineus* populations. The gene flow between the Acehnese *A. testudineus* population and the Indonesian fish population is related.

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