Molecular Characterization of Anguilla sp. with Special Notes on Its Population Genetic in the Rivers of Cilacap Central Java, Indonesia

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Abstract. Taxonomic status of Anguilla species in the rivers of Cilacap is vital for further study, such as population genetics and evolutionary history. Taxonomic status, population genetic, and evolutionary histories of Anguilla can be assessed using single genetic marker, such as cytochrome oxidase 1 gene. This study aimed to determine taxonomic status, genetic diversity and connectivity, and evolutionary history of Anguilla populations in the rivers of Cilacap. Anguilla samples were collected from Doplang-Serayu and Segara Anakan watersheds. Sequence homology test to the conspecific sequence in GenBank proved that all samples from Doplang-Serayu watershed were genetically identified as Anguilla bicolor (98.23 to 100% homology). The decision was supported by monophyly between samples and their conspecific references. Anguilla bicolor from both watersheds had high haplotype (gene) diversity. Amova and Fst analysis proved that no genetic difference (p=0.623) was observed between Doplang-Serayu and Segara Anakan watersheds, indicated panmixing. Haplotype network proved that both populations were evolved from two primitive ancestors. This study concluded that Anguilla bicolor was the only freshwater eel observed in the rivers of Cilacap. Anguilla bicolor in the rivers of Cilacap has high genetic diversity but no genetic differentiation was observed among populations. Anguilla bicolor population in the rivers of Cilacap evolved from two primitive ancestors.

Key words: ancestor, barcoding, diversity, eel, evolution, population

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INTRODUCTION

The coastal area of southern Central Java is among the well-known source of freshwater eel from the genus Anguilla (Sugeha et al., 2008; Fahmi et al., 2015a). Cilacap is among the regencies in the southern part of Central Java, famous as a sampling site for anguillid studies (Fahmi et al., 2015b; Dahrudin et al., 2016; Wibowo et al., 2021). Several studies reported a different number of Anguillid species from the rivers of Cilacap. Sugeha (2008) reported that two species of Anguilla from Citanduy estuary Cicacap; A. interioris and Anguilla spp. Fahmi et al. (2015) and Mutmainah et al. (2016) found A. bicolor in Segara Anakan estuary Cilacap. Nuryanto et al. (2020) reported Anguilla bicolor from Cibeureum and Sapuregel watersheds Cilacap. Dahrudin et al. (2016) reported two species of anguillid; A. bicolor and A. marmorata. The previous studies collected Anguilla samples from the riverine ecosystem emptied in the Segara Anakan estuary.

Cilacap Regency has several estuaries of big rivers, such as the Serayu estuary, Doplang estuary, and on the east border of the regency is located Lukulo estuary (Taufiq et al., 2021). A previous study by Wibowo et al. (2021) reported Anguilla bicolor from the Serayu River, and Taufiq et al. (2021) reported A. bicolor from all estuaries in Cilacap Regency. However, two morphometric ratio data suggested two species of A. bicolor and Anguilla bicolor Pacifica are present in the rivers of Cilacap, especially from Buntong Marshes (Doplang River estuary) (Taufiq et al., 2021). Moreover, it has also been suggested that A. bengalensis lives in the Serayu River (Krismono, personal communication). These questionable reports need further validation, such as using genetic character (Taufiq et al., 2021).

The taxonomic status of eels from the rivers of Cilacap, especially from the Doplang watershed,
is essential for further studies, such as the study of population genetics of the species. Those studies (taxonomic status determination and population genetics) of freshwater eels from Doplang watersheds can be done using a single genetic marker, such as the cytochrome c oxidase (COI) gene (Nuryanto et al., 2019). Previous studies proved the COI gene's reliability as a barcode marker in species delineation (Imtiaz et al., 2017; Afrand et al., 2018; Bingpeng et al., 2018; Ahmed et al., 2019; Bhagawati et al., 2020; Ude et al., 2020). Several other studies demonstrated that the COI gene is also a good marker for population genetic studies in broad range animal phyla (Artamonova et al., 2018; Padmavathi and Srinu, 2019; Limmon et al., 2020; Roesma et al., 2020).

Earlier studies reported broad scales of population genetic of Anguilla bicolor with some samples were collected from Segara Anakan estuary (Famhi et al. 2015a; 2015b; Norarfan et al., 2021). At the same time, Nuryanto et al. (2020) had evaluated the genetic diversity of A. bicolor from Sapuregel and Cibeureum watersheds which are also emptied in Segara Anakan estuary. However, all studies focused on assessing genetic diversity A. bicolor either from limited sampling sites or limited sampling size in the rivers of Cilacap. Those studies could not provide reliable data for making policy in Anguilla fisheries management in the Cilacap Regency. Meanwhile, according to Indonesia Law No. 23 2014, Regional Governments have the authority to manage the resources in their territory, and good management needs comprehensive data about their resources.

Taxonomic status and small-scale population genetic are meaningful for local management (Adrian-Kalchhauser et al., 2016; Rosche et al., 2016; Breton et al., 2018). Previous studies proved that several population genetic data is essential for fisheries management and conservation (Marie et al., 2016; Scribner et al., 2016; Wennerstrom et al., 2016). The data inferred from the genetic research of Anguilla in the rivers of Cilacap are taxonomic status, connectivity among tributaries, and evolutionary history of Anguilla, which is vital to ensure that Anguilla in all riverine systems should be managed separately or as a single entity. Previous studies stated that genetic unit should be managed as single fisheries management or single conservation unit (Pearse, 2016; Pavlova et al., 2017; Beacham et al., 2020).

This study aimed to determine taxonomic status, genetic diversity and connectivity, and evolutionary history of Anguilla populations in the rivers of Cilacap, Central Java, Indonesia. The data is essential for Anguilla fisheries management in the region.

METHODS

Sampling sites

Anguilla samples were collected from Serayu (C) and Doplang (D) River watersheds in the District of Adipala, Cilacap Regency. Additional 25 Anguilla bicolor sequences were retrieved from GenBank, where the samples were collected from the

![Figure 1. Southern areas of Cilacap Regency indicate sampling sites (red colors are current sampling, blue colors are sampling sites where the sequences retrieved from GenBank, and map modified from Google Earth online version 2021)](image-url)
Cibeureum River (A) District of Kedungreja and Sapuregel River (B) District of Kawunganten, Cilacap Regency (Figure 1).

**Sampling**
A sampling of *Anguilla* specimens in Adipala (Serayu and Doplang Rivers) was conducted on June 2021 with the help of local fishermen. Silver and yellow *Anguilla* was collected using a trap made of a plastic tube connected to a triangular net. This study set up the traps facing downstream for 24 hours from 16:00 to 08:00 the next day. We performed sampling efforts five times and obtained 46 individuals of freshwater eel from four sites in Doplang and Serayu.

**DNA isolation, cytochrome c oxidase 1 gene amplification, and sequencing**
The DNA of *Anguilla* samples was extracted using Chelex®100 (Walsh et al., 1991), with incubation times were modified become 6 hours (Nuryanto et al., 2019). The cytochrome c oxidase 1 (COI) gene fragments were amplified using a pair of primers. Forward primer and reverse primer FishR2 (Ward et al., 2005). Marker amplifications were conducted in PEQLAB Primus 25 thermal cycler with the following condition. The Pre-denaturation step was performed at 95°C for 5 minutes. The cycles consisted of denaturation step at 94°C for 1 minute, annealing at 52°C for 1.5 minutes, and elongation step at 72°C for 1 minute. The cycles were repeated 35 times and terminated by a final extension at 72°C for five minutes. The PCR reactions have consisted of 2 µM of MgCl$_2$, 1X buffer solution, 4 µM dNTPs, 4 pM of each primer, 2 µl template DNA solutions, and 2 U polymerase enzymes. The final volume of PCR reactions we adjusted to 50 µl by adding RNAase and DNase pure water. The quality of the PCR products was checked qualitatively by 1% agarose electrophoresis migration. Qualified PCR products were shipped to 1st BASE Asia for sequencing. Marker sequencing was conducted using a big dye terminator following the Sanger method.

**Sequence editing**
The COI gene sequence was edited and trimmed manually using Bioedit package 7.0 (Hall, 2011). Functional coding sequences were determined by submitting the sequence to an online version of ORF finder (https://www.ncbi.nlm.nih.gov/orffind et/) with search parameters consisted of minimal ORF length of 300 nucleotides, genetic code mitochondrial vertebrate, and start codon ATG and alternative initiation codons. Afterward, the ORF sequences were verified through a basic local alignment search tool (BLAST) with format option graphical overview, link-out, sequence retrieval, and CDS feature. The study aligned query and subject sequences manually to ensure that stop codons were absent along the sequences. Multiple sequences alignments were conducted using ClustalW as part of Bioedit package version 7.0 (Hall, 2011).

**Taxonomic status of *Anguilla* samples from Doplang and Serayu watersheds**
The present study determined the taxonomic status of the samples by comparing the sequences of the samples to the closely related taxa available in GenBank or BOLD data. This study determined the taxonomic category of the samples based on percent identity, genetic divergence, genetic distances, and monophyly. Percent identity value of 97% or 3% sequence divergence (Meyer & Paulay, 2005; Ratnasingham & Hebert, 2007) was determined as the genetic threshold for species border. Some studies reported intraspecific genetic divergences between 3% and 5% in some species but with some exceptions (Jeffery et al., 2011; Higashi et al., 2011). The Kimura 2-parameter (K2P) genetic distance and branch length of 0.05 were also used as additional species border parameters. The K2P genetic distances were calculated using MEGAX software (Kumar et al., 2018). Monophyly between samples and references species was estimated from the phylogenetic tree. The tree was reconstructed based K2P substitution model using maximum likelihood and maximum parsimony algorithms in MEGAX (Kumar et al., 2018). The topology was polarized through outgroup comparison and bootstrap replication. A sum of 1000 pseudo-replication was used during the calculation. *Anguilla bicolor pacifica* (LC548772), *Anguilla marmorata* (KU692251), and *A. celebesensis* (AP007239) were selected as the outgroup species. The uses of these three species were expected to have the best polarization on tree branching patterns because they have overlap geographic distribution.

**Historical demography of *Anguilla bicolor* in the rivers of Cilacap**
Demographic history of *Anguilla bicolor* in the rivers of Cilacap was conducted using two types of samples. The first type was fresh samples which
were collected from Doplang and Serayu Rivers. Second type was a meta-analysis of *Anguilla bicolor* sequences retrieved from GenBank (the specimens were collected from Cibereum and Sapuregel watersheds Cilacap (Nuryanto et al., 2020). A neutrality test to examine the hypothesis of neutral evolution of the COI gene was conducted using the Fu’s Fs and Tajima’s D test (Tajima, 1989) with 10,000 simulated samples using Arlequin 3.5 program (Excoffier & Lischer, 2010). A significant result of the neutrality test indicates either bottleneck or population expansion. Therefore, the Roger test was further performed to test demographic population expansion. We performed these tests with 10,000 replications in Arlequin 3.5 program (Excoffier & Lischer, 2010).

**Genetic diversity and population genetic**

Population genetic study was also included fresh samples and meta-analysis of sequences from GenBank. All samples from Doplang and Serayu watersheds were combined after preliminary AMOVA analysis proved that both watersheds did not show a significant genetic difference. *Anguilla bicolor* sequences retrieved from GenBank (Accession number MT883612-MT883625), collected from Cibereum and Sapuregel Rivers Cilacap Regency, were also treated as a single population because they were not genetically significantly different (Nuryanto et al., 2020) and referred to as Segara Anakan population. Genetic diversity was estimated using haplotype h (Nei 1987) and nucleotide π (Nei and Jin, 1989) diversity. We performed the analysis in

| No. | Sample Code | Identity (%) | Expect Value | Reference Species | Accession Number |
|-----|-------------|--------------|--------------|-------------------|------------------|
| 1   | KRC01       | 99.83        | 0.00         | *Anguilla bicolor* | MG675613         |
| 2   | KRC02       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 3   | KRC03       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 4   | KRC04       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 5   | KRC05       | 99.84        | 0.00         | *Anguilla bicolor* | KU692247         |
| 6   | KRC08       | 99.52        | 0.00         | *Anguilla bicolor* | MT883622         |
| 7   | KRC10       | 100.0        | 0.00         | *Anguilla bicolor* | MW078538         |
| 8   | KRY01       | 99.67        | 0.00         | *Anguilla bicolor* | KU692247         |
| 9   | KRY02       | 99.74        | 0.00         | *Anguilla bicolor* | KM875505         |
| 10  | KRY03       | 99.84        | 0.00         | *Anguilla bicolor* | MG675613         |
| 11  | KRY04       | 100.0        | 0.00         | *Anguilla bicolor* | MW078538         |
| 12  | KRY06       | 98.35        | 0.00         | *Anguilla bicolor* | AP007236         |
| 13  | KRY08       | 100.0        | 0.00         | *Anguilla bicolor* | KU692247         |
| 14  | KRY09       | 100.0        | 0.00         | *Anguilla bicolor* | KM875504         |
| 15  | KLD01       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 16  | KLD02       | 99.85        | 0.00         | *Anguilla bicolor* | MF612058         |
| 17  | KLD03       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 18  | KLD04       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 19  | KLD05       | 99.84        | 0.00         | *Anguilla bicolor* | MG675613         |
| 20  | KLD06       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 21  | KDL07       | 100.0        | 0.00         | *Anguilla bicolor* | KU692247         |
| 22  | KLD08       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 23  | SRY01       | 100.0        | 0.00         | *Anguilla bicolor* | KU692247         |
| 24  | SRY02       | 99.55        | 0.00         | *Anguilla bicolor* | MG675613         |
| 25  | SRY03       | 99.84        | 0.00         | *Anguilla bicolor* | KU692247         |
| 26  | SRY04       | 100.0        | 0.00         | *Anguilla bicolor* | MG675613         |
| 27  | SRY05       | 100.0        | 0.00         | *Anguilla bicolor* | KU692247         |
| 28  | SRY06       | 98.23        | 0.00         | *Anguilla bicolor* | KU692247         |
| 29  | SRY07       | 99.68        | 0.00         | *Anguilla bicolor* | MG675613         |
| 30  | SRY08       | 99.35        | 0.00         | *Anguilla bicolor* | KF182304         |
Arlequin 3.5 (Excoffier and Lischer, 2010). Population comparison was conducted by calculating of variance component among populations and fixation index (Fst) value using analysis of molecular variance (AMOVA) in Arlequin 3.5 (Excoffier and Lischer, 2010) based on Kimura 2-parameter substitution model without gamma shape parameter. The substitution model and gamma shape parameter were estimated using MEGAX (Kumar et al., 2018). Significant values were obtained from a comparison with a p-value for both parameters.

Evolutionary history of Anguilla bicolor in the rivers of Cilacap

The present study predicted the evolutionary history of Anguilla bicolor from the rivers of Cilacap by creating a haplotype network. A haplotype network was reconstructed by involving the outgroup species, as previously explained. The network was calculated using median-joining and drawn using NETWORK 10.2 free software (Bandelt et al., 1999).

RESULT AND DISCUSSION

Taxonomic status of Anguilla specimen from Doplang and Serayu watersheds

Sequence similarity test to the conspecific reference in GenBank showed that Anguilla samples from the rivers of Cilacap have percent identities ranged from 98.23% to 100%, with the expected value of 0.00 (Table 1). In other words, all samples have sequence divergences to their conspecific references ranged between 0.00% and 1.77%. This study considered high percent identities to top ten hits conspecific references. The data were not showed in Table 1 because all the top ten hits were Anguilla bicolor.

According to the data in Table 1, all samples have a percent identity value above a pre-determined threshold value of 97%, or sequence divergences were below 3% to their conspecific sequence in the GenBank. Genetic identity of 97% is commonly used as species border in animal barcoding or maximum sequence divergence 3% (Ratnasingham & Hebert, 2007; Hubert et al., 2010; Ko et al., 2013). The present study observed that the genetic identity of all samples was above 97% means that all samples are genetically identified as Anguilla bicolor. Since the survey was conducted in the rivers that faced the Indian Ocean, our samples were Anguilla bicolor bicolor. There is a clear geographic separation between A. bicolor bicolor and A. bicolor pacifica (Minegishi et al., 2012; Norarfan et al., 2021). Previous studies were also reported high genetic identity between samples Anguilla bicolor to their reference species, although the populations were collected from different geographic locations (Dahrudin et al., 2016; Nuryanto et al., 2020; Norarfan et al., 2021; Wibowo et al., 2021). Previous studies showed that intraspecific genetic identity might be below 97% depending on the fish groups (Mat Jaafar et al., 2012; Pereira et al., 2013; Ratnasingham & Hebert, 2013; Lim et al., 2016; Nuryanto et al., 2018).

All freshwater eel samples collected from Doplang-Serayu Rivers formed a monophyletic clade even compared to Anguilla bicolor pacifica with branches length-scale less than 0.010 (Figure 2). The separation between our samples and A. bicolor pacifica was supported by high bootstrap values (92/94/94 for MP/ML/NJ algorithms, respectively). The monophyly clade of Anguilla bicolor showed in Figure 2 strengthens that our samples were Angiola bicolor bicolor. Therefore, all samples were phylogenetically identified as Anguilla bicolor from subspecies A. bicolor bicolor. A previous study has argued that individuals can be considered a single species when produced from a single evolutionary process and form a monophyletic clade (Xu et al., 2015; Nuryanto et al., 2017). The present of Anguilla bicolor in Serayu Rivers was also reported by Wibowo et al. (2021). In contrast, Taufiq et al. (2021) was not sure about the present of A. bicolor and A. bicolor pacifica from Doplang Marshes and suggest to do a molecular validation. The present study, further validate that only one Anguilla species inhabit Doplang watershed, that is Anguilla bicolor.

The present study found a similar species of freshwater eel, as reported by Wibowo et al. (2021), from the upper part of the Serayu River. The similarity strengthens the available data that Anguilla bicolor is the dominant freshwater eel species in the southern areas of Indonesia, especially in the south of Cilacap. Similar species was also reported from Segara Anakan Cilacap (Fahmi et al., 2015a) and from Cibeureum and Sapuregel River Cilacap (Nuryanto et al., 2020). The finding of Anguilla bicolor from Serayu and Doplang tributaries in the District of Adipala was reasonable because the rivers have ecological characteristics best for Anguilla bicolor to live. Low land river systems are typical habitats for A. bicolor. That species is rarely found in highland river systems (Froese & Pauly, 2021).

All specimens which were sampled further upstream from the estuary were genetically identified as A. bicolor. On the one hand, the finding corroborated a report that glass eels of A. bicolor were the most abundant specimens obtained.
Figure 2. Maximum parsimony tree showing monophyly between samples from DOplang_Serayu watershed with their conspecific references obtained from GenBank.
by Widodo et al. (2021). On the other hand, our result contradicted what Wibowo et al. (2021) reported about yellow/silver eel abundance, which was extremely rare during their survey, while this study was easily found as A. bicolor specimens on yellow/silver eel stages.

**Historical demography**

Using Tajima's D and Fu's Fs analysis, historical demography analysis rejected neutral evolution of the used marker COI gene for both sampling sites. However, these results could not explain either selection or population expansion had occurred. Roger test and mismatch distribution analysis indicated that both populations had undergone sudden population expansion as characterized by the acceptance of sudden population expansion and mismatch distribution hypothesis (p>0.05; Table 2). In the case of the Anguilla bicolor population in the southern Cilacap, population expansion could be corroborated by a pandemic period. Where lockdown, large-scale social restriction, and restriction on community activities implemented by the Indonesian government have reduced eel fishing activities in the rivers of Cilacap for two years.

**Genetic diversity and population genetic**

Multiple sequence alignment resulted in a 528 bp fragment of the COI gene. A total of 45 sites out of 508 bp fragments were polymorphic with has polymorphism level of 8.52%. It means that the used marker has the most common allele with a frequency of 91.48%. The obtained frequency diversity of Anguilla bicolor. Wibowo et al. (2021) was summarized overall genetic diversity across Indo-Pacific Region. This study summarized overall genetic from several tributaries in the rivers of Cilacap. Nevertheless, this study found that the haplotype diversity of Anguilla bicolor in the rivers of Cilacap has a similar pattern to the genetic diversity of A. bicolor at their geographic ranges, even using a different molecular marker (Fahmi et al. 2015a) used cytochrome b.

**Table 2.** Tajima D, Fu Fs, the sum of square deviation (SSD), Harpending’s Raggedness index (HRi), and p-values for all the parameters.

| Sampling Site               | D     | p-value | Fs   | P-value | SSD | p-value | HRi  | p-value |
|-----------------------------|-------|---------|------|---------|-----|---------|------|---------|
| Rivers of Cilacap           | -     | 0.001   | -    | 0.000   | 0.014ns | 0.657 | 0.030ns | 0.722 |
| Doplang-Serayu              | 2.147*** | 0.000 | 19.356*** | 0.000 | 0.022ns | 0.452 | 0.044ns | 0.481 |
| Segara Anakan               | -1.884* | 0.014 | -7.593**  | 0.000 | 0.098ns | 0.583 | 0.036ns | 0.515 |

**Table 3.** Sampling site, sample size (N), number haplotype (nhp), number of polymorphic sites (nps), polymorphism (p), haplotype (h), and nucleotide (π) diversity.

| Sampling site                      | N  | nhp | nps | p (%) | h          | π          |
|------------------------------------|----|-----|-----|------|------------|------------|
| Freashwater ecosystem of Cilacap    | 55 | 28  | 45  | 8.52 | 0.849±0.047 | 0.0069±0.0039 |
| Doplang_Serayu                     | 30 | 18  | 36  | 6.82 | 0.887±0.052 | 0.0086±0.0049 |
| Segara Anakan                      | 25 | 13  | 16  | 3.03 | 0.810±0.079 | 0.0047±0.0029 |
This study observed slightly higher genetic diversity of *A. bicolor* than the previous report by Fahmi et al. (2015a). The difference was because of the different numbers of sampling sites and sample sizes. The present study analyzed *Anguilla bicolor* specimens from four tributaries in the rivers of Cilacap with 55 specimens, while Fahmi et al. (2015a) was analyzed five individuals from Segara Anakan. In some cases, different sample sizes resulted in varying levels of genetic diversity, with small sample sized tend to have low genetic diversity (Joshi et al., 2013; Nuryanto et al., 2019).

Nucleotide diversity of *A. bicolor* from the rivers of Cilacap was low with the value of 0.0068 ±0.0039. Low level of haplotype diversity was reasonable because only 45 polymorphic sites were observed out of 528 base pairs fragment in all pairwise comparison among 55 sequences. Keragaman nukleotida yang rendah diamati pada gen COI *A. bicolor* dari keduas DAS karena hanya memiliki 45 situs polimorfik dari 508 situs yang dianalisis. Nei (1987) mencatat bahwa nilai keanekaragaman nukleotida di bawah 1% dikategorikan sebagai tingkat rendah. Namun, pada penelitian ini diperoleh nilai keragaman yang lebih tinggi dari penelitian Nuryanto et al. (2020) padaikan sidat dari Sungai Cibeureum dan Sapuregel, Segara Anakan.

Haplotype diversity (h) value within the population was 0.887±0.052 for the Doplang-Serayu population, and 0.810±0.079 indicated that both populations have high gene diversity. The high gene diversity proved that *Anguilla bicolor* population in both populations carries variable alleles. In contrast, both populations have low nucleotide diversity (π) with the value of 0.0086±0.0049 for the Doplang-Serayu population and 0.0047±0.0027. The low nucleotide diversity could indicate that the COI gene in *Anguilla bicolor* has a slow mutation rate of their nucleotide. This study compared both populations and found that the Doplang-Serayu population has slightly higher than that in the Segara Anakan population in both genetic diversity parameters. It is because both populations are affected by differential pressure. During sampling periods, the population in the Segara Anakan was continuously harvested although the pandemic period, while in the Doplang-Anakan population, zero exploitation was observed. Genetic diversity of values as observed within this study was a normal phenomenon because each animal has their-own evolutionary rate and trend. High and low genetic diversity has been reported in a wide range of animals, starting from invertebrate to vertebrate, including fish (Dung et al., 2013; Song et al., 2013; Zhang et al., 2014; Liu et al., 2017; Parmaksiz & Eksi, 2017).

Variance component and Fst value proved that Doplang-Serayu population and Segara Anakan populations were genetically not different (p=0.623; Table 4). The finding was contradictory to a general acceptance that river populations show genetic differentiation among river systems (Hughes et al., 2009). Even significant genetic divergence could be observed within a river system without physical barrier (Kanno et al., 2011). The difference could be that *Anguilla bicolor* is a catadromous that moves from river to ocean to spawn (Arai, 2020). The spawning ground of *Anguilla bicolor* is southwest of Sumatra, which makes individuals from different rivers meet. In contrast, Hughes et al. (2009) and Kanno et al. (2011) were utilized potamodromous species with limited dispersal along the river. Therefore, it was reasonable that Hughes et al. (2009) observed significant genetic differences among river systems, and Kanno et al. (2011) found a significant genetic difference within the river system of brook trout.

No significant genetic difference between Doplang-Serayu and Segara Anakan populations indicated that *Anguilla bicolor* in the rivers of Cilacap is a pan mixing population. This argument was based on that all river systems emptied to the Indian Ocean, where spawning ground of *Anguilla bicolor* was predicted to be located in the Indian Ocean southwest of Sumatera (UNEP-WMC, 2011).

| Source of variation | df | Sum of Squares | Variance Components | Percentage of Variation |
|---------------------|----|----------------|---------------------|-------------------------|
| Among populations   | 1  | 0.341          | -0.003 Va<sub>ns</sub> | -0.74                  |
| Within populations  | 53 | 22.587         | 0.426 Vb            | 93.48                  |
| Total               | 54 | 22.927         | 0.423               |                         |
| Fixation Index FST  |  = | -0.007<sup>ns</sup> |                     |                         |
| p-value             |  = | 0.623 ± 0.005 |                     |                         |

ns= non-significant.
No genetic difference among river populations was reported in *H. Nemurus* populations in Java Island (Nuryanto et al., 2019). Conversely, significant genetic differences were observed in various river fish populations (Dung et al., 2013; Pavesi et al., 2011). Previous studies using microsatellite markers, which are believed to be more sensitive than that COI gene, were also showed variable results. On the one hand, studies using microsatellites showed no genetic difference among fish populations (Damerau et al., 2012; Basharat et al., 2016). On the other hand, previous studies using microsatellites were showed significant genetic differences among populations (Abbas et al., 2017; Bartáková et al., 2013; Esa & Abdul Rahim, 2013; Yan et al., 2017). This study and previous studies indicated that genetic differentiation among populations is a complex phenomenon whatever genetic markers are utilized. The current and previous studies proved that genetic differentiation among nature population is a complex phenomena (Parmaksiz and Eksi 2017; Achrem et al. 2017; Parmaksiz 2019; Ariyaraphong et al. 2021).

**Evolutionary history of *Anguilla bicolor* in the rivers of Cilacap**

The present study estimated the evolutionary history of *Anguilla bicolor* in the rivers of Cilacap through haplotype network reconstruction (Figure 3). The network was reconstructed based on the sequence obtained during the present study, sequences from other regions inside and outside Indonesia (GenBank). We compared the samples to other *Anguilla* species present in Indonesia waters to infer the most primitive haplotype. It can be observed in the network (Figure 4) that *Anguilla bicolor* populations in the rivers of Cilacap consisted of two haplogroups (A and B). The individual formed both haplotypes from both Doplang-Seray and Segara Anakan samples and *Anguilla bicolor* from other regions. Within Haplogroup A, haplotype 1 (H1) was referred to as a primitive ancestor. This study determined H1 as a primitive ancestor of *A. bicolor* in the rivers of Cilacap because formed by 28 individuals from the rivers of Cilacap and outside Cilacap (Yogyakarta and India). The primitive haplotype in haplogroup B was haplotype 4 (H4). It was determined based on number of individuals and geographic distribution of the samples. It has been stated that older haplotypes could be determined by a high number of individuals within the haplotype and the wide geographic distribution of the haplotype (Barasa et al. 2014). Haplotype 4 was consisted of 15 individuals from Doplang-Serayu, Segara Anakan, Kebumen, Sukabumi, Aceh, Malaysia, and India).

Two haplogroups of *A. bicolor* in the rivers of Cilacap might indicate that *A. bicolor* populations in the rivers of Cilacap originated from two spawning sites. The first spawning site was suggested to be located in the southwest of Sumatera and off east Madagaskar (UNEP-WMC, 2015; Wibowo et al., 2021). Our haplotype network might corroborate the hypothesis by showing that haplotype 8, collected from Kenya, and it was placed together in haplogroup B.

**Conservation**

Population panmixing and a limited maternal ancestor that contributes to form *A. bicolor*

![Figure 4](image_url). Haplotype network indicate *Anguilla bicolor* populations in Freashwater ecosystem of Cilacap evolved from a single primitive ancestor
populations in the rivers of Cilacap has vital implications for fisheries management and conservation of freshwater eels in the rivers of Cilacap, Central Java, Indonesia. Panmixia might imply that *Anguilla bicolor* in all river systems in Cilacap Regency must be considered a single genetic conservation unit. Populations with high genetic connectivity indicated by no genetic differences have high conservation values (Estradivari et al., 2017) and should be managed as a single conservation unit (Vargas et al., 2016). Nevertheless, more samples and additional genetic markers are needed to provide highly reliable genetic data as the basis for conservation and management.

This study provides more comprehensive data about taxonomic status and population genetic of *Anguilla bicolor* in the rivers of Cilacap, Central Java, Indonesia. The data essential for conserving and management of *Anguilla bicolor* fisheries in Cilacap Regency, especially the capture fisheries sector. In term of conservation, this data suggests that *Anguilla bicolor* in the rivers of Cilacap should be treated as a single conservation unit.

**CONCLUSION**

This study concluded that *Anguilla bicolor* is the only freshwater eel found in the Serayu and Doplang tributaries District of Adipala, Cilacap Regency, Central Java, Indonesia. *Anguilla bicolor* in the rivers of Cilacap showed high genetic diversity but no genetic difference among tributaries. *Anguilla bicolor* population in the rivers of Cilacap are evolved from two primitive ancestors. This finding implied that *Anguilla bicolor* populations in the rivers of Cilacap should be managed as a single genetic conservation unit.

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