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

Agus Nuryanto[*], Sri Riani, Dian Bhagawati, Elly Tuti Winarni, Farida Nur Rachmawati

Faculty of Biology, Universitas Jenderal Soedirman, Indonesia

[*]Corresponding Author: agus.nuryanto@unsoed.ac.id

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

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 Bunton 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,
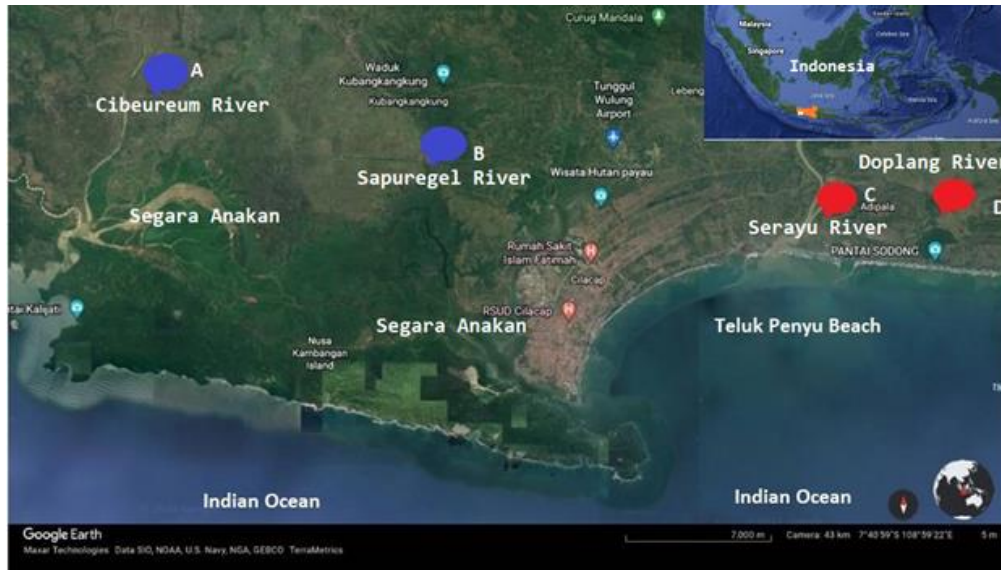
Figure 1. Southern areas of Cilacap Regency indicate sampling sites (red colors are current sampling, blue colors are sampling sites where the sequences retrieved from GenBank, and map modified from Google Earth online version 2021)

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 esturay. 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

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 DNAase 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 er/) 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 Cibeureum 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 Cibeureum 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

Table 1. Sample code, percent identity, expect value, reference species and its accession number

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 bocilor*.

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 (Dahruddin 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 *Anguila 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
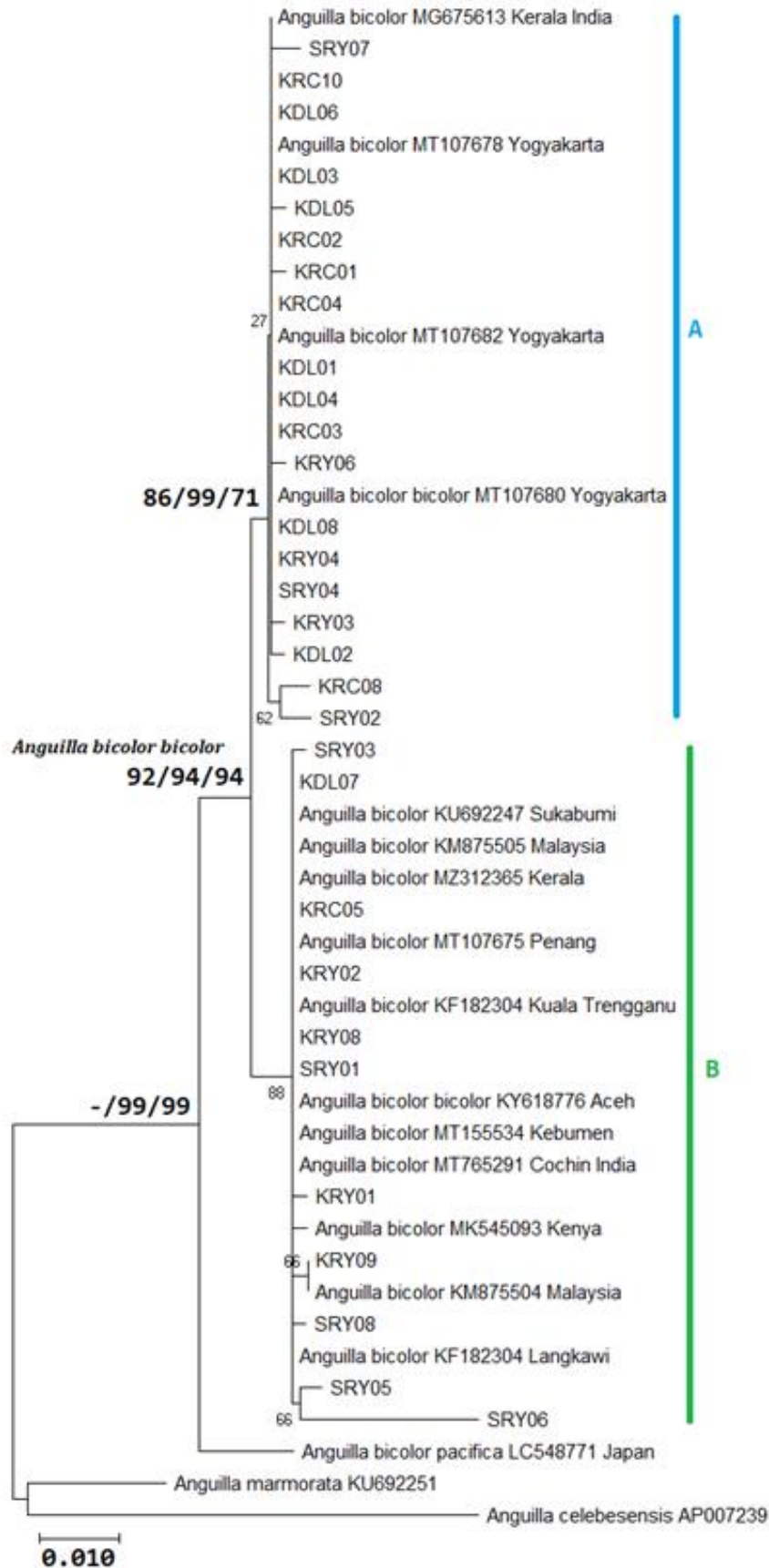
Figure 2. Maximum parsimony tree showing monophyly between samples from DOplang_Serayu watershed with their conspecific references obtained from GenBank

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

value indicates that the COI gene of *Anguilla bicolor* utilized in this study was polymorphic. Previous literature stated that a locus could be called polymorphic when the most common allele has a maximum frequency of 95% (Nuryanto et al., 2019).

Overall haplotype diversity of COI gene in *Anguilla bicolor* from the rivers of Cilacap was $0,822 \pm 0,074$ (Table 3). The value indicated that A. bicolor has high haplotype diversity because the obtained value was inside the range as reported by Nei (1987). In his paper, Nei (1987) stated that haplotype diversity ranged between 0.08 and 1 is considered high diversity. This finding was similar to previous studies that report *Anguilla bicolor* has high haplotype diversity (Fahmi et al., 2015; Wibowo et al., 2021). However, the comparison to those studies didn't match congruence wholly because of different sampling site coverage. Firstly, the comparison to Fahmi et al. (2015) did not show conformity because they used other markers. The present study utilized the COI gene, while Fahmi et al. (2015a) used cytochrome b.

Nevertheless, this study and Fahmi et al. (2015a) showed a similar level of haplotype

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	-2.147[***]	0.001	-19.356[***]	0.000	0.014[ns]	0.657	0.030[ns]	0.722
Doplang-Serayu	-1.884[*]	0.014	-7.593[**]	0.003	0.022[ns]	0.452	0.044[ns]	0.481
Segara Anakan	-1.497[*]	0.050	-6.548[***]	0.000	0.009[ns]	0.583	0.036[ns]	0.515

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

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

al., 2015a).

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 kedua 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) pada ikan 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±00.27. 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-Serayu 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,

Table 4. Result amova

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

ns= non-significant

2015).

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 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*
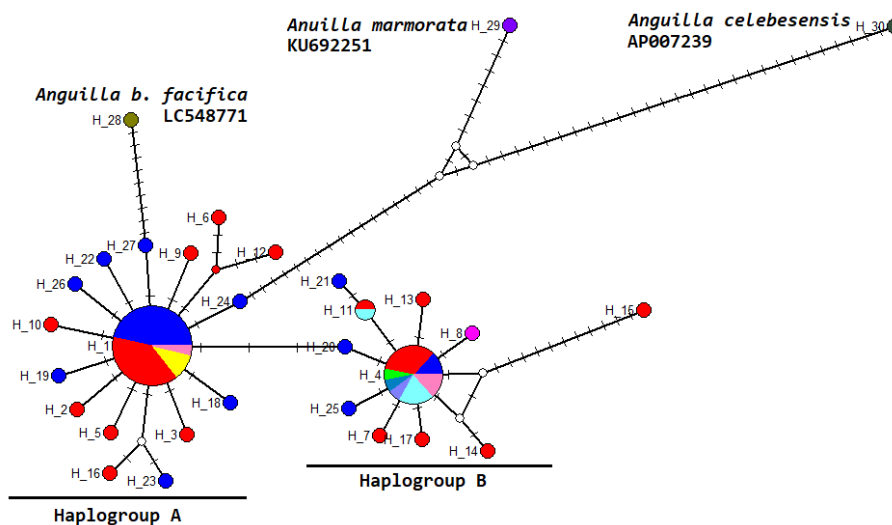

Figure 4. Haplotype network indicate Anguilla bicolor populations in Freashwater ecosystem of Cilacap evolved from a single primitive ancestor

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.

ACKNOWLEDGMENT

REFERENCES

Abbas, K., Xiaoyun, Z., & Weimin, W. (2017). Microsatellite markers reveal genetic differentiation of Chinese dojo loach Misgurnus anguillicaudatus in the Yangtze River basin. *Turkish Journal of Fisheries and Aquatic Sciences* 17:1167-1177.

Achrem, M., Skuza, L., Kirczuk, L., Domagala, J., Pilecka-Rapacz, M., & Czerniawski, R. (2017). Assessment of genetic variability in common whitefish from the catchment area of the Oder River using microsatellite markers. *Acta Biologica* 24: 5-13.

Adrian-Kalchhauser, I., Hirsch, P. E., Behrmann-Godel, J., N'guyen, A., Watzlawczyk, S., Gertzen, S., Borcherding, J., Burkhardt-Holm, P. (2016). The invasive bighead goby Ponticola kessleri displays large-scale genetic similarities and small-scale genetic differentiation in relation to shipping patterns. *Molecular Ecology* 25: 1925-1943.

Afrand, M., Sourinejad, I., Fazeli, S. A. S., & Akbarzadeh, A. (2018). Identification of Engraulids (*Encrasicholina punctifer* and *E. pseudoheteroloba*) using the COI gene as a DNA barcoding marker. *Journal of Aquatic Ecology* 7(3): 39-47.

Ahmed, Md. S., Chowdhury, Md. M. K., & Nahar, L. (2019). Molecular characterization of small indigenous fish species (SIS) of Bangladesh through DNA barcodes. *Gene* 684: 53-57.

Arai, T. (2020). Ecology and evolution of migration in the freshwater eels of the genus *Anguilla* Schrank, 1798. *Helion* 6: e05176. https://doi.org/10.1016/j.heliyon.2020.e05176

Ariyaraphong, N., Laopichienpong, N., Singchat, W., Panthum, T., Ahmad, S. F., Jattawa, D., Duengkae, P., Muangmai, N., Suwanasopee, T., Koonawootrittriron, S., & Srikulnath, K. (2021). High-level gene flow restricts genetic differentiation in dairy cattle populations in Thailand: insights from large-scale Mt D-Loop sequencing. *Animals* 11: 1680.

Artamonova, V.S., Kolmakova, O. V., Kirillova, E. A., & Makhrov, A. A. (2018). Phylogeny of salmonoid fishes (Salmonoidei) based on mtDNA COI gene sequences (barcoding). *Contemporary Problem in Ecology* 11(3): 271-285.

Bandelt, H. J., Foster, P., & Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16(1): 37-48.

Bartáková et al., (2013). Strong population genetic structuring in an annual fish, *Nothobranchius furzeri*, suggests multiple savannah refugia in southern Mozambique. *BMC Evolutionary Biology* 13: 196.

Beacham, T. D., Wallace, C., Jonsen, K., McIntosh, B., Candy, J. R., Rondeau, E. B., Moore, J-S., Bernatchez, L., & Withler, R. (2020). Accurate estimation of conservation unit contribution to coho salmon mixed-stock fisheries in British Co

lumbia, Canada, using direct DNA sequencing for single nucleotide polymorphis ms. *Canadi an Journal of Fisheries and Aquatic Science* 77: 1302-1315.

Breton, T. S., Nettleton, J. C., O'Connell, B., & Ber tocci, M. (2018). Fine-scale population genetic structure of sugar kelp, Saccharina latissima (La minariales, Phaeophyceae), in eastern Maine, USA. *Phycologia* 57(1): 32-40.

Barasa, J. E., Abila, R., Grobler, J. P., Dangasuk, O. G., & Njahira, M. N. 2014. Genetic diversity an d gene flow in *Clarias gariepinus* from Lakes V ictoria and Kanyaboli, Kenya. *African Journal of Aquatic Science* 39(3): 287-293. DOI: 10.298 9/16085914.2014.933734

Basharat, H., Ghafoor, A., Chavhan, A., Zafar, M. M., Abbah, K., Tabasum, J., & Parveen, R. (2016). Microsatellite markers revealed poor genetic structure of *Wallago attu* in Punjab, Pakistan. *International Journal of Life Sciences* 4(3): 385-393.

Bhagawati, D., Winarni, E. T., & Nuryanto, A. (2020). Molecular barcoding reveals the existence of mole crabs *Emerita emeritus* in North Coast of Central Java. *Biosaintifika* 12 (1): 104-110

Bingpeng, X., Heshan, L., Zhilan, Z., Chunguang, W., Yanguo, W., & Jianjun, W. (2018). DNA barcoding for identification of fish species in the Taiwan Strait. *PLoS ONE* 13(6): e0198109

Dahruddin, H., Hutama, A., Busson, F., Sauri, S., Hanner, R., Keith, P., Hadiaty, R., & Hubert, N. (2016). Revisiting the ichthyodiversity of Java and Bali through DNA barcodes: taxonomic coverage, identification accuracy, cryptic diversity and identification of exotic species. *Molecular Ecology Resources* DOI: 10.1111/ 1755-0998.12528

Damerau, M., Matschiner, M., Salzburger, W., & Hanel, R. (2012). Comparative population genetics of seven notothenioid fish species reveals high levels of gene flow along ocean currents in the southern Scotia Arc, Antarctica. *Polar Biology* 35: 1073-1086.

Dung, D. T., Hop, N. T., Thaenkham, U., & Waik agul, J. (2013). Genetic differences among Viet namese *Haplorchis taichui* populations using the COI genetic marker. *Journal of Helmint hology* 87: 66-70.

Esa & Abdul Rahim, 2013. Genetic structure and preliminary findings of cryptic diversity of the Malaysian mahseer (Tor tambroides Valen ciennes: Cyprinidae) inferred from mitochon drial DNA and microsatellite analyses. *BioMed Research International* 2013: Article ID 17098

0, 14 pages. ttp://dx.doi.org /10.1155/2013/170 980

Estradivari, Handayani, C. N. N., Daniel, D., & Mus tofa, A. (2017). Marine protected area network design: A case study of Maluku Province. *Coas tal and Ocean Journal* 1(2): 135-146.

Excoffier, L. & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10(3): 564–567. https://doi.org/10. 1111/j.1755-0998.2010.02847.x

Fahmi, M. R., Solihin, D. D., Soewardi, K., Pou youd, L., & Berrebi, P. (2015a). Molecular phy logeny and genetic diversity of freshwater *Angu illa* eels in Indonesian waters based on mitocho ndrial sequences. Life and Environment 65(3): 139-150.

Fahmi, M. R., Solihin, D. D., Shao, Z., Pouyoud, L., & Berrebi, P. (2015b). Population genetic struc ture of the tropical eel *Anguilla bicolor* in Indo nesian waters based on microsatellite markers.

Froese, R. & Pauly, D. (eds). (2021). Fishbase. Wor ld Wide Web Electronic Publication. www.fish base.org, version (06/2021)

Hall, T.A. (2011). BioEdit: an important software for molecular biology. *Nucleic Acids Symposium Series* 41: 95-98

Higashi, R., Tsukagoshi, A., Kimura, H., & Kato, K. (2011). Male dimorphism in a new interstitial species of the genus *microloxoconcha* (Podoco pida: Ostracoda). *GERF Bulletin of Biosciences* 2(1): 60-61.

Hubert, N., Delieu-Trottin, E., Irisson, J. O., Meyer, C., & Planes, S. (2010). Identifying coral reef fish larvae through DNA barcoding: A test case with the families Acanthuridae and Holocentri dae. Molecular Phylogenetics and Evolution 55: 1195-1203.

Hughes, J. M., Schmidt, D. J., & Finn, D. S. (2009). Genes in streams: using DNA to understand the movement of freshwater fauna and their river ine habitat. *Bioscience* 59: 573-583.

Imtiaz, A., Nor, S. A. M., Naim, D. MD. (2017). Progress and potential of DNA barcoding for species identification of fish species. *Biodiver sitas* 18(4): 1394-1405.

Kanno, Y., Vokoun, J. C., & Leetcher, B. H. (2011). Fine-scale population structure and riverscape genetics of brook trout (*Salvelinus fontinalis*) distributed continuously along headwater chan nel networks. *Molecular Ecology* 20: 3711-3729.

Ko, H. L., Wang, Y. T., Chiu, T. S., Lee, M. A., Leu, M. Y., Chang, K. Z., Chen, W. Y., & Shao, K. T. (2013). Evaluating the accuracy of morphological identification of larval fishes by applying DNA barcoding. *PLoSONE* 8 (1): 253451.

Jeffery, N. W., Elias-Guttierrez, M., & Adamowicz, S. J. (2011). Species diversity and phylogeographical affinities of the Branchiopoda (Crustacea) of Churchill, Manitoba, Canada. *PLoS ONE* 6 (5): e18364.

Joshi, J., Salar, R. K., Banerjee, P., Upasna, S., Tantia, M. S., & Vijh, R. K. (2013). Genetic variation and phylogenetic relationships of Indian buffaloes of Uttar Pradesh. *Asian-Australasian Journal of Animal Sciences* 26(9): 1229-1236.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35 (6): 1547-1549.

Kusbiyanto, Bhagawati, D., & Nuryanto, A. (2020). DNA barcoding of crustacean larvae in Segara Anakan, Cilacap, Central Java, Indonesia using cytochrome c oxidase gene. *Biodiversitas* 21(10): 4878-4887.

Lim, H. C., Muchlisin, Z. A., Pulungan, C. P., de Bruyn, M., & Nor, S. A. M. (2016). DNA barcoding reveals high cryptic diversity of the freshwater halfbeak Genus *Hemirhamphodon* from Sundaland. *PLoS ONE* 11(9): e0163596

Limmon, G., Delrieu-Trottin, E., Patikawa, J., Rijoly, F., Dahruddin, H., Busson, F., Steinke, D., & Hubert, N. (2020). Assessing species diversity of Coral Triangle artisanal fisheries: A DNA barcode reference library for the shore fishes retailed at Ambon harbor (Indonesia). *Ecology and Evolution* 10: 3356–3366.

Liu, X., 2017. Genetic structure and diversity of *Nodularia douglasiae* (Bivalvia: Unionida) from the middle and lower Yangtze River drainage

Marie, A. D., Lejeusne, C., Karapatsiou, E., Cuesta, J. A., Drake, P., Macpherson, E., Bernatchez, L., & Rico, C. (2016). Implications for management and conservation of the population genetic structure of the wedge clam Donax trunculus across two biogeographic boundaries. *Scientific Report* 6: 39152 | DOI: 10.1038/srep39152.

Mat Jaafar, T. N. A., Taylor, M. I., Mohd Nor, S. A., de Bruyn, M., & Carvalho, G. R. (2012). DNA barcoding reveals cryptic diversity within commercially exploited Indo-Malay Carangidae (Teleostei: Perciformes). *PLoS ONE* 7(11): e49623.

Meyer, C. P. & Paulay, G. (2005). DNA Barcoding: Error rates based on comprehensive sampling. PLoS Biology 3(12): e422

Minegishi, Y., Gagnaire P-A., Aoyama, J., Bosc, P., Feunteun, E., Tsukamoto, K., & Berrebi, P. (2012). Present and past genetic connectivity of the Indo-Pacific tropical eel Anguilla bicolor. *Journal of Biogeography* 39: 408-420.

Mutmainah, D., Honda, S., Suryati N. K., & Prosantoso, B. I. (2016). Understanding the current status of Anguillid eels fisheries in Southeast Asia. *Fish & People* 14(3): 19-25.

Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M. & Jin, L. (1989) Variances of the average numbers of nucleotide substitutions within and between populations. *Molecular Biology and Evolution* 6: 290–300.

Norarfan, A. F., Mokti, S. T. A., Taha, H., Amin, M., Ali, M., & Arai, T. (2021). DNA barcoding of a tropical anguillid eel, Anguilla bicolor (Actinopterygii: Anguilliformes), in Indo-Pacific region and notes on its population structure. *Zoologia* 38: e59332

Nuryanto, A., Pramono, H., & Sastranegara, M. H. (2017). Molecular identification of fish larvae from East Plawangan of Segara Anakan, Cilacap, Central Java, Indonesia. *Biosaintifika* 9(1): 33-40.

Nuryanto, A., Pramono, H., Kusbiyanto, Ghifari, M. I., & Andareswari, N. (2018). Barcoding of fin clip samples revealed high exploitation of *Plectropomus leopardus* in the Spermonde Archipelago. *Biosaintifika* 10(3): 629-635.

Nuryanto, A., Komalawati, N., & Sugiharto. (2019). Genetic diversity assessment of *Hemibagrus nemurus* from rivers in Java Island, Indonesia using COI gene. *Biodiversitas* 20(9): 2707-2717.

Nuryanto, A., Bhagawati, D., Rukayah, S., Rahayu, D. R. U. S., & Wibowo, D.D. (2020). Molecular barcoding reveals possible existence of sympatric species of *Emerita emeritus* in South Coast of Cilacap Central Java. *IOP Conference Series: Earth and Environmental Science* 593: 012014.

Padmavathi, P. & Srinu, G. (2019). Genetic divergence and phylogenetic analysis of fish fauna from Lake Kolleru based on COI sequences. *Current Trends in Biotechnology and Pharmacy* 13 (2): 173-189.

Parmaksiz, A. & Eksi, E. (2017). Genetic diversity of the cyprinid fish *Capoeta trutta* (Heckel, 1843) populations from Euphrates and Tigris rivers in Turkey based on mtDNA COI sequences. *Indian Journal of Fisheries* 64(1):

18-22.

Parmaksiz, A. (2019). Population genetic diversity of yellow barbell (*Carasobarbus luteus*) from Kueik, Euphrates and Tigris Rivers based on mitochondrial DNA D-loop sequences. *Turkish Journal of Fisheries and Aquatic Sciences* 20(1): 79-86.

Pavesi L., De Matthaeis, E., Tiedemann, R., & Ketmaier, V. (2011). Temporal population genetics and COI phylogeography of thesandhopper *Macarorchestia remyi* (Amphipoda: Talitridae) .*Zoological Studies* 50(2): 220-229.

Pavlova, A., Beheregaray, L. B., Coleman, R., Gilligan, D., Harrisson, K. A., Ingram, B. A., Kearns, J., Lamb, A. M., Lintermans, M., Lyon, J., Nguyen, T. T. T., Sasaki, M., Tonkin, Z., Yen, J. D. L., & Sunnucks, P. (2017). Severe consequences of habitat fragmentation on genetic diversity of an endangered Australian freshwater fish: a call for assisted gene flow. *Evolutionary Applications* 10:531–550

Pearse, D. E. (2016). Saving the spandrels? Adaptive genomic variation in conservation and fisheries management. *Journal of Fish Biology* 89: 2697-2716.

Pereira, L. H. G., Hanner, R., Foresti, F., & Olivera, C. (2013). Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna? *BMC Genetic* 14:20.

Ratnasingham, R. & Hebert, P. D. N. (2013). A DNA-based registry for all animal species: the barcode index number (BIN) system. *Plos ONE* 8(8): e66213. doi:10.1371/journal.pone.0066213

Ratnasingham, R. & Hebert, P. D. N. (2007). BOLD: The Barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes* 7: 355-364.

Roesma, D. E., Tjong D. H., & Aidil, D. R. (2020). Phylogenetic analysis of transparent gobies in three Sumatran lakes, inferred from mitochondrial Cytochrome Oxidase I (COI) gene. *Bio diversitas* 21(1): 43-48.

Rosche, C., Duka, W., Hensen, I., Mraz, P., Hartmann, M., Muller-Scharer, H., & Lachmuth S. (2016). The population genetics of the fundamental cytotype-shift in invasive *Centaurea stoebe* s.l.: genetic diversity, genetic differentiation and small-scale genetic structure differ between cytotypes but not between ranges. *Biological Invasions* 18: 1895-1910

Scribner et al., (2016). Applications of genetic data to improve management and conservation of river fishes and their habitats. *Fisheries* 41(4): 174-188.

Song, L. M., Munian, K., Rashid, Z. A., & Bhassu, S. (2013). Characterisation of Asian snakehead murrel *Channa striata* (Channidae) in Malaysia: an insight into molecular data and morphological approach. *The Scientific World Journal* 2013: Article ID 917506, 16 pages. http://dx.doi.org/10.1155/2013/917506.

Sugeha, H. Y., Suharti, S. R., Wouthuyzen, S., & Sumadhiharga, K. (2008). Biodiversity, distribution and abundance of the tropical anguillid eels in the Indonesian waters. *Marine Research Indonesia* 33(2): 129-137.

Tajima, F. (1989). Statistical method for testing then mutation hypothesis by DNA polymorphism. *Genetics* 123: 585-595.

Taufiq-Spj, N., Hutabarat, J., Trianto, A., Sugianto, D. N., Santosa, G. W., Pratikto, I., Ario, R., Indarjo, A., & Suryono, A. (2021). Morphometric distribution of Java eel *Anguilla* sp. caught from different estuaries of Central Java. *IOP Conf. Series: Earth and Environmental Science* 750: 012042.

Ude, G. N., Igwe, D. O., Brown, C., Jackson, M. , Bangura, A., Chieze, V., Ozokonkwo-Alor, O., Ihearahu, O. C., Chosen, O., Okoro, M., Ene, C. Chieze, V., Unachukwu, M., Onyia, C., Acquaah, G., Ogbonna, J., & Das, A. (2020). DNA bar coding for identification of fish spe cies from freshwater in Enugu and Anambra States of Nigeria. *Conservation Genetics Re sources* 12: 643-658.

UNEP-WCMC. (2015). Preliminary overview of the genus *Anguilla*. UNEP-WCMC, Cambridge.

Vargas, S. M., Jensen, Ho, S. Y. W., Mobaraki, A., Broderick, D., Mortimer, J. A., Whiting, S. D., Miller, J., Prince, R. I. T., Bell, I. P., Hoenner, X., Limpus, C. J., Santos, F. R., & Fitz Simmons, N. N. (2016). Phylogeography, genetic diversity, and management units of hawksbill turtles in the Indo-Pacific. *Journal of Heredity* 107(3): 199-213.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B* 360(1462): 1847-1857.

Wennerstrom, L., Jansson, E., & Laikre, L. (2016). Baltic Sea genetic biodiversity: Current knowledge relating to conservation management. *Aquatic Conservation: Marine and Freshwater Ecosystems* 27: 1069-1090.

Wibowo, A., Nicolas Hubert, N., Dahruddin, H., Steinke, D., Suhaimi, R. A., Samuel, Atminarso, D., Anggraeni, D. P., Trismawanti, I., Baumgartner, L. J., & Ning, N. (2021). Assessing

temporal patterns and ppecies composition of glass eel (Anguilla spp.) cohorts in Sumatra and Java using DNA barcodes. *Diversity* 13: 193. https://doi.org/10.3390/ d13050193

Xing, B., Chen, X., Zhang, Z., Sun, R., Xiang, P., Lin, M., & Wang, C. (2020). Genetic identification of ophichthid fishes through DNA barcoding. *Mitochondrial DNA Part B* 5(2): 1970-1974.

Xu, X., Liu, F., Chen, J., Li, D., & Kuntner, M. (2015). Integrative taxonomy of the primitively segmented spider genus *Ganthela* (Araneae: Mesothelae: Liphistiidae): DNA barcoding gap agrees with morphology. *Zoological Journal of Linnean Society* 175(2): 288-306. DOI: 10.1111/zoj.12280

Yan, R-J., Zhang, G-R., Guo, X-Z., Ji, W., Chen, K-C., Zou, G-W., Wei, K-J., & Gardner, J. P. A. (2017). Genetic diversity and population structure of the northern snakehead (*Channa argus* Channidae: Teleostei) in central China: implications for conservation and management. *Conservation Genetics*. DOI 10.1007/s10592-017-1023-x

Zhang, G-H., Yuan, Z-J., Zhang, C-X., Yin, K-S., Tang, M-J., Guo, H-W., Fu, J-Y., & Xiao, Q. (2014). Detecting deep divergence in seventeen populations of tea geometrid (*Ectropis obliqua* Prout) in China by COI mtDNA and Cross-Breeding. *PLoS ONE* 9(6): e99373.

Zhang, J. B. & Hanner, R. (2011). DNA barcoding is a useful tool for the identification of marine fishes from Japan. *Biochemical Systematics and Ecology* 39: 31-42.