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Short Communication

Molecular Identification of Elvers (*Anguilla* spp.) from River Estuaries in Central Java, Indonesia Using DNA Barcoding Based on mtDNA CO1 Sequences

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Abstract

An inventory of the presence and diversity of *Anguilla* spp. needs to be carried out as a basis for sustainable resource management. Conventional techniques based on morphological characteristics—are often less effective considering the morphological characteristics of *Anguilla* spp. are very identical and not so many can be observed, especially at the young eel stage. DNA-based molecular identification can be a way to determine diversity and phylogeny for conservation and inventory purposes. This research aimed to determine the diversity and phylogeny of *Anguilla* spp. obtained at the estuaries of Serayu River in Cilacap, Luk Ulo River in Kebumen, and Jali River in Purworejo in Central Java, Indonesia. A total of 10 samples were taken from each research location were analyzed morphologically, then it is known that there are 4 morphologically different samples at each research location so that a total of 12 samples were taken for molecular analysis. MEGA was used to construct the phylogenetic trees via Neighbour Joining (NJ) algorithms using the Kimura 2-parameter model with uniform rates and obtained by 1000 bootstraps replication. There are four species of *Anguilla* spp. identified morphologically, namely *Anguilla bicolor bicolor*, *Anguilla bicolor pacifica*, *Anguilla obscura*, and *Anguilla australis*. The species of *Anguilla* spp. that is molecularly identified is *Anguilla bicolor bicolor* with an identity percentage of > 98%. All samples are also known to be related to *Anguilla bicolor bicolor* identified in other countries.

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1. Introduction

Eels (*Anguilla* spp.) is a fish with a distinct lifecycle due to its catadromous life phase, in which it begins its life in the sea, grows and develops in freshwater, and then returns to the sea to breed when it reaches maturity (Matondo and Ovidio, 2018; Arai and Taha, 2021). This fish is found all over the world, with 16 species and three subspecies identified, all of which can be split into two groups based on the climate in which they inhabit, namely tropical and temperate eels (Arai, 2016; Jellyman, 2021). Fahmi (2015), using semi- multiplex PCR analysis, found six species and one subspecies of eels in Indonesia, namely, *Anguilla bicolor bicolor, Anguilla bicolor pacifica, Anguilla marmorata*, *Anguilla interioris*, *Anguilla bengalensis*, *Anguilla celebensis,* and *Anguilla borneensis*. Arai (2016) added *Anguilla obscura* to the list, bringing the total eels' species in Indonesia to seven species with one subspecies. There is no report about diversity of elvers molecularly in estuaries of Serayu River in Cilacap, Luk Ulo River in Kebumen, and Jali River in Purworejo. Nevertheless, the population and diversity of eels have been reported to decline due to climate change and overexploitation (Matondo and Ovidio, 2018; Dekker, 2016). Therefore, it is necessary to take an inventory of the presence and diversity of eels as a basis for sustainable resources management, as well as conservation efforts.

Eels' identification is frequently based solely on morphology (Huyen *et al*., 2020). However, due to the great similarity of morphological features, identifying eel species only on the basis of morphology will be extremely difficult, especially at the juvenile stage (elvers) in the tropics (Arai, 2016). The lack of visible and limited morphology in elvers creates a problem in which morphological identification alone is sometimes insufficient. The identification of this genus is also influenced by its similar morphological characters and wide geographical distribution. This is due to the fact that scientific names frequently change over time for a variety of reasons, resulting in incompatibility between scientific names submitted to public databases and their use unless the database is updated (Silvergrip, 2009). The species identification used in this paper will be based on Silvergrip (2009) and CITES, with updated subspecies names. This method is an approach that uses a DNA database to identify all known species, as well as a tool to provide criteria for new species recognition. The molecular approach can also identify individual characteristics. If these characters' information is available, germplasm variation can be accessed effectively and efficiently for conservation and further research (Anggraeni *et al*., 2008). Molecular identification of eels has been successfully conducted (Arai *et al*., 2020; Alter *et al*., 2015; Wibowo *et al*., 2021). Furthermore, detection of the presence and diversity of eel revealed by environmental DNA analysis (Kasai *et al*., 2021; Takahashi *et al*., 2021).

Because some tropical eels are overexploited, threatened with extinction, and sold commercially around the world as food, species identification is critical for management and monitoring (Fahmi *et al*., 2013). The high potential of eels necessitates research on the genetic diversity of eel species in Indonesian waters as one of the first steps in conservation efforts. Identification to the species level is performed using molecular analysis in the elvers phase because identification to the same level using morphological characters alone is known to be difficult due to character similarity (Watanabe *et al*., 2009). Therefore, this research aims to determine the diversity and phylogeny of *Anguilla* spp. obtained at the estuaries of Serayu River in Cilacap, Luk Ulo River in Kebumen, and Jali River in Purworejo in Central Java, Indonesia as a basis for fishery management and conservation efforts of Anguilla spp.

2. Materials and Methods

2.1 Study Site

This study was conducted in the southern region of Central Java, specifically in Estuary of Jali River (Kebumen Regency), Luk Ulo River (Purworejo Regency), and Serayu River (Cilacap Regency) (Figure 1). The data was collected in October 2021 by buying the fish from the local fishermen, then preserved with 96% alcohol solution and analyzed at the Sub Laboratory of Aquatic Ecology, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada.

2.2 Method

2.2.1 Morphological identification

Elver were collected from the fisherman at the research site. Morphological characteristics were identified by comparing the characteristics of the samples with the Anguillidae identification reference

book (Silvergrip, 2009; Tesch and Thorpe, 2003). The species characteristics in Tesch and Thorpe (2003) and Watanabe *et al.* (2008) are used to estimate morphological species. The characteristics observed included fin position, pattern, number of vertebrae, and the percentage of the ratio of the distance between the dorsal fin and anal fin compared to the total length using the formula (D-A/PT) %, where D is the length of the dorsal fin in centimeters, A is the length of the anal fin in centimeters, and PT is the total length of the fish in centimeters.

2.2.2. Molecular identification

DNA extraction of elvers (*Anguilla* spp.) was carried out using a DNA extraction kit following the Favorgen Tissue Genomic DNA Extraction Mini Kit protocol. The stages of DNA isolation include tissue dissociation, lysis, DNA binding/DNA binding, DNA washing, and elution. Following that, agarose gel electrophoresis was then visualized to determine the presence of isolated DNA. The fragment of Cytochrome c oxidase subunit I was amplified by PCR combi block machine from Matra Biometra Germany. The PCR amplification was performed by inserting 7.5µl PCR mix + 1µl F primer + 1µl R primer + 1µl DNA template + 4.5µl NFW into the PCR tube. The primers used were FISH F1(5'-TCAACCAACCACAAAGACATTGGCAC-3') and R1 (5' TAGACTTCTGGGTGGCCAAAGAATCA-3') targeting the DNA nucleus (Ward *et al*., 2005). The DNA amplification procedure was modified by Ward *et al.* (2005) and Peninal *et al.* (2017), with predenaturation at 94°C for one minute, followed by 34 denaturation cycles at 94°C for 34 seconds, annealing at 45°C for 40 seconds, extension at 72°C for one minute, and final extension at 72°C for 10 minutes.

2.3 Data Analysis

The DNA amplification results were sequenced by sending the DNA PCR results to PT. Genetika Science. The nucleotide base sequence data obtained is subsequently processed using MEGA-X software. Nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program which can be accessed on the National Center for Biotechnology Information (NCBI) web page.

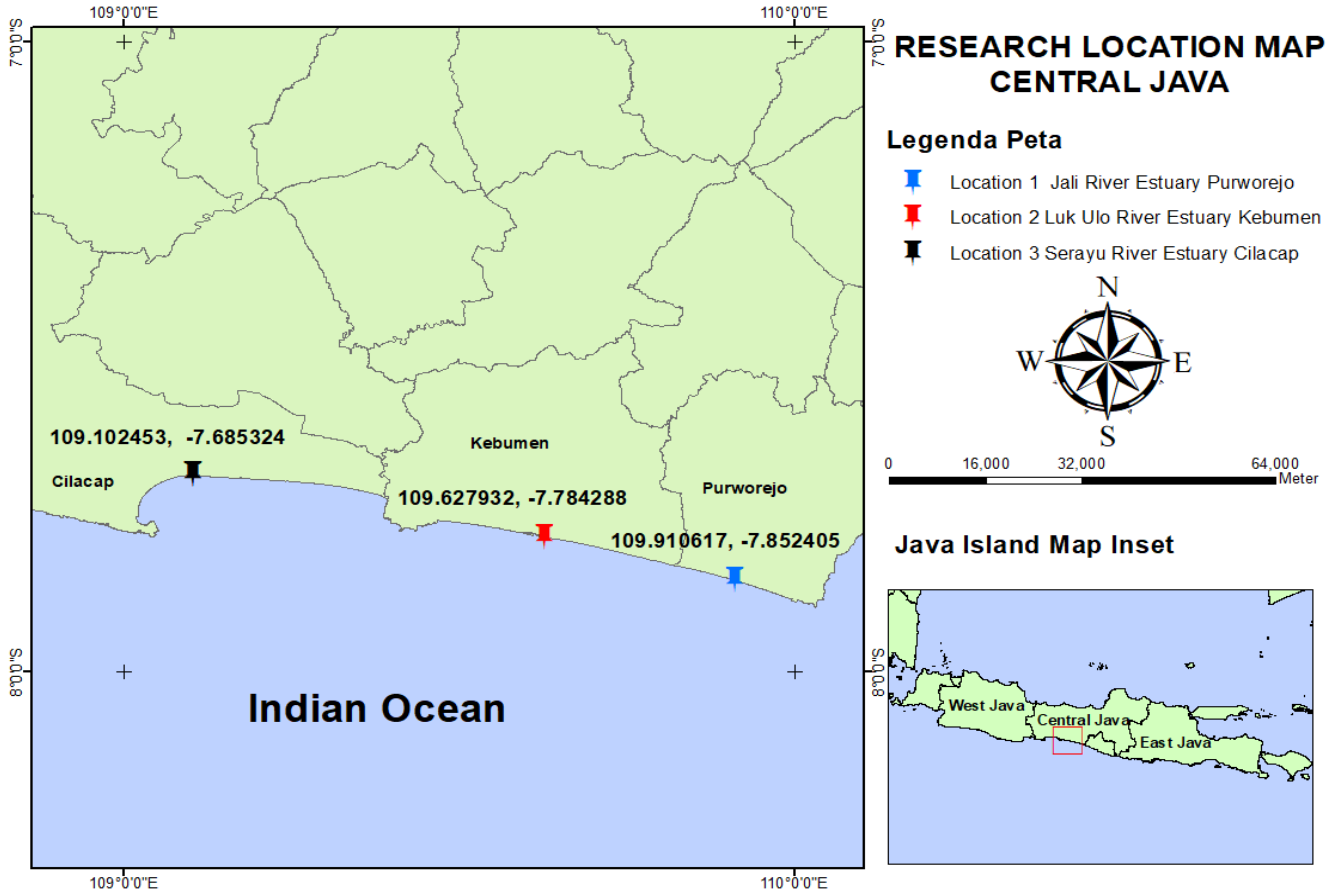

Figure 1. Study site where the elver of *Anguilla* spp. were collected

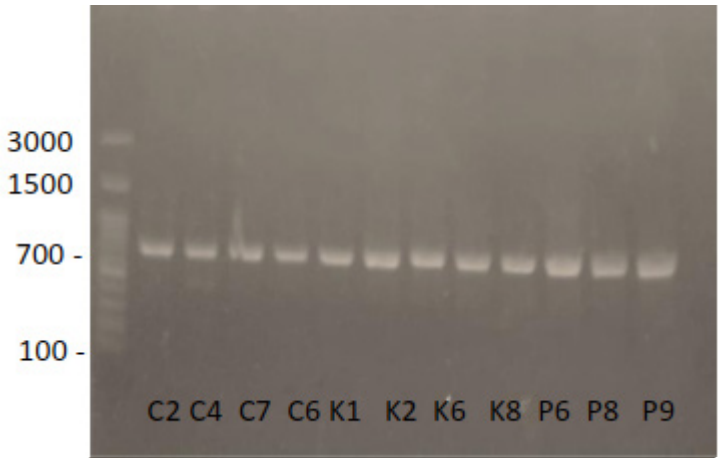

Figure 2. Electrophoresis results

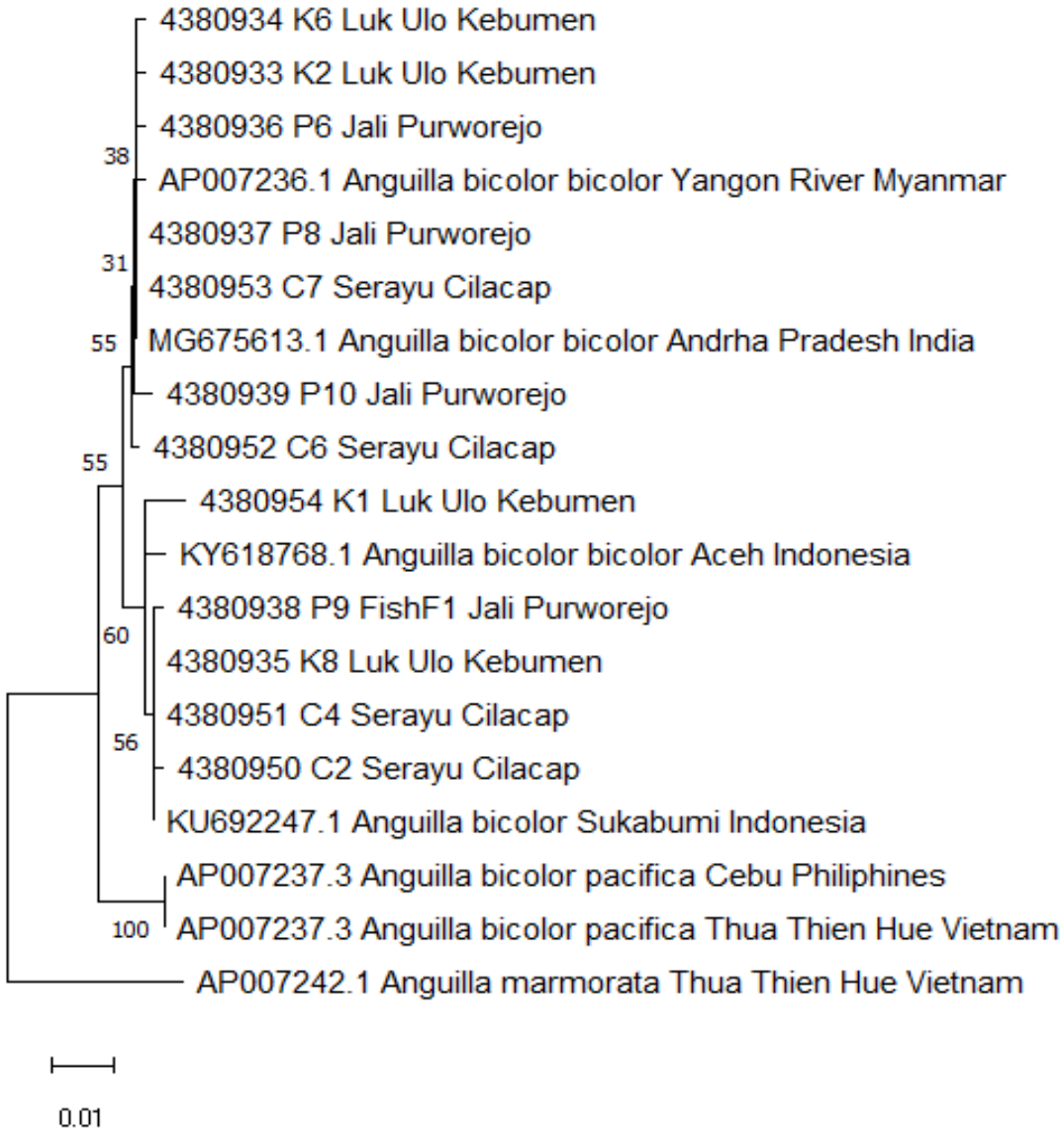

Figure 3. The phylogenetic tree of *Anguilla* spp. in Estuary of the Jali River, Luk Ulo River, and Serayu River, Central Java, Indonesia with eel species found in several other countries using the Neighbor-Joining(NJ) method using the Kimura 2-parameter mode

The species name and nucleotide sequence similarity level of Anguilla spp. samples were determined by comparing the BLAST results with GenBank data at NCBI. The MEGA-X software was used to create the phylogenetic tree by aligning the DNA sequences acquired with the DNA sequences on GenBank. The sequences of eels from Purworejo, Cilacap and Kebumen were submitted to the GenBank database with accession numbers 4380936 - 4380939, 4380950 - 4380953, 4380933-4380935 and 4380954, respectively. In addition to these twelve sequences, some COI sequences of *Anguilla* spp. specimens from other localities that were found in the GenBank were also included in the analysis: Aceh in northern Sumatra of Indonesia (KYKY618768.1), Sukabumi in western Java of Indonesia (KU692247), Andhra Pradesh of India (MG675613.1), Yangon of Myanmar (AP007236.1), Cebu of Philippines (AP007237), and Thua Thien Hue of Vietnam (AP007242.1 and AP007237.3) (Nonarfan *et al.*, 2021; Huyen *et al.*, 2020). MEGA was used to construct the phylogenetic trees via Neighbour Joining (NJ) algorithms using the Kimura 2-parameter model with uniform rates and obtained by 1000 bootstraps replication.

3. Results and Discussion

3.1 Morphological Identification of Elver Anguilla spp.

Based on the results of morphological identification, it is known that four different species were identified morphologically from a total of 30 samples (Table 1). *Anguilla bicolor bicolor*, *Anguilla bicolor pacifica*, *Anguilla obscura*, and *Anguilla australis* were recognized as physically distinct species in the Estuary of Jali River, Luk Ulo River, and Serayu River. All of the sample fin positions discovered are parallel and have a plain skin pattern (Table 1). This narrowed the estimation of sample species to four, namely *A. bicolor pacifica*, *A. bicolor bicolor*, *A. obscura*, and *A. australis*. The discovery of species in the three river estuaries is unusual because the habitat of the species *A. bicolor pacifica*, *A. australis*, and *A. obscura* is not in the waters south of Java (Arai, 2016). According to Watanabe *et al.* (2004), when it comes to geographic distribution, which is as significant as morphology, the most likely species to be found in the Serayu, Luk Ulo and Jali River Estuaries is only *A. bicolor bicolor*.

Environmental variables and the phase of *Anguilla* spp. caused very comparable and short-spanning traits among the four species to be discovered. Elver is a transition from young eels to juveniles, and it can cause a morphological identification bias. The same results were also found by Amrullah *et al.* (2019) that *A. marmorata* in Sulawesi has differences in morphological characteristics namely the body height at the front pectoral fin; body height at the front dorsal fin; body height at front anus; dorsal fin height; anal fin height; and caudal fin height.

3.2 Molecular Identification of Elver Anguilla spp.

Based on the electrophoresis visualization of the CO1 gene from the PCR process, four samples of *Anguilla* spp. from the Jali River Estuary in Purworejo, four samples from the Luk Ulo Purworejo Estuary, and four samplesfrom the Serayu River Estuary, Cilacap showed the success of the DNA amplification process using two different pairs of primers, namely FISH R1 and FISH F1 at an annealing temperature of 45°C which was indicated by the presence of luminescence in all samples. The results of electrophoresis of the CO1 gene PCR results from samples K1, K2, K6, K8, P6, P8, P9, P10, C2, C4, C6, and C7 on irradiation using UV light showed a size of about ± 700 bp (Figure 2).

According to Domingues (2017), the annealing temperature must be optimal to maximize the amplification of the target genomic sequence while minimizing the risk of non-specific amplification. The annealing temperature can be tweaked if necessary by increasing or reducing it by a few degrees until the desired outcome is achieved. The use of primers FISH R1 and FISH F1 at an annealing temperature of 44°C succeeded in bringing out the amplified target DNA band around the 700 bp (1kb marker) marker band position. This suggests that in this investigation, the nucleotide length that can be amplified using FISH R1 and FISH F1 primers is ± 700 bp. As in earlier research, the emergence of the target DNA band at this number suggests that amplification of the target gene in the genus Anguilla was successful (Abdullah *et al*., 2018; Ward *et al*., 2005). These findings back up the assertion of Sasmito *et al.* (2014) that the properties of the primers used, and their suitability have a significant impact on the success of PCR.

From the samples identified molecularly, there is only one species observed, i.e., *Anguilla bicolor bicolor* (Table 2). The query cover with the highest percentage is shown by sample K6 with a value of 99% from Kebumen, P10 with a value of 99% from Purworejo, and C7 with a value of 99% from

Table 1. The results of morphological identification of elvers in the Estuary of Jali River, Luk Ulo River, and Serayu River, Central Java, Indonesia referred to Tesch and Thorpe (2003) and Watanabe *et al*. (2008).

Location	Code	Fin Position	(D-A/PT)%	Skin pattern	Total number of vertebrae	Suspect species
Jali River, Purworejo	P-01	Parallel	0.6	Plain	93	*A. bicolor bicolor*
	P-02	Parallel	5.7	Plain	108	*A. obscura*
	P-03	Parallel	0	Plain	89	*A. bicolor pacifica*
	P-04	Parallel	0.7	Plain	102	*A. bicolor bicolor*
	P-05	Parallel	0	Plain	110	*A. bicolor pacifica*
	P-06	Parallel	0.6	Plain	75	*A. bicolor bicolor*
	P-07	Parallel	0	Plain	107	*A. bicolor pacifica*
	P-08	Parallel	0	Plain	112	*A. bicolor pacifica*
	P-09	Parallel	4.3	Plain	104	*A. obscura*
	P-10	Parallel	2.8	Plain	115	*A. australis*
Luk Ulo River, Kebumen	K-01	Parallel	0.7	Plain	101	*A. bicolor bicolor*
	K-02	Parallel	5.5	Plain	108	*A. obscura*
	K-03	Parallel	0	Plain	101	*A. bicolor pacifica*
	K-04	Parallel	0	Plain	97	*A. bicolor pacifica*
	K-05	Parallel	0.7	Plain	91	*A. bicolor bicolor*
	K-06	Parallel	2.8	Plain	107	*A. australis*
	K-07	Parallel	0.6	Plain	94	*A. bicolor bicolor*
	K-08	Parallel	0	Plain	92	*A. bicolor pacifica*
	K-09	Parallel	0.6	Plain	94	*A. bicolor bicolor*
	K-10	Parallel	0.7	Plain	99	*A. bicolor bicolor*
Serayu River, Cilacap	C-01	Parallel	0	Plain	98	*A. bicolor pacifica*
	C-02	Parallel	4.9	Plain	111	*Anguilla obscura*
	C-03	Parallel	0	Plain	105	*A. bicolor pacifica*
	C-04	Parallel	0.7	Plain	114	*A. bicolor bicolor*
	C-05	Parallel	0	Plain	92	*A. bicolor pacifica*
	C-06	Parallel	0	Plain	97	*A. bicolor pacifica*
	C-07	Parallel	1.3	Plain	107	*A. australis*
	C-08	Parallel	0.6	Plain	91	*A. bicolor bicolor*
	C-09	Parallel	0	Plain	94	*A. bicolor pacifica*
	C-10	Parallel	0	Plain	99	*A. bicolor pacifica*

Cilacap. The highest percent identity is indicated by samples with codes K2 and K8 with a value of 100% originating from Kebumen,P8 with a value of 100% originating from Purworejo, and C4 and C6 with a value of 100% originating from Cilacap, while the lowest percent identity is indicated by sample with code K1 originating from Kebumen with a value of 99.21%.

The results of molecular identification differ significantly from those of morphological identification. The species *A. bicolor bicolor* was molecularly identified in samples C6, K8, and P8, but identified as *A. bicolor pacifica* from morphological identification. Meanwhile, molecular identification results from samples C2, K2, and P9 showed the species *A. bicolor bicolor*, but the morphological identification showed the species *A. obscura*. Furthermore, molecular identification results from samples C7, K6, and P10 showed the species *A. bicolor bicolor*, but

Table 2. The results of molecular identification on elvers in the Estuary of Jali, Luk Ulo River, and Serayu River, Central Java, Indonesia

No	Sample code	Identified species	*Query Cover*	*Percent Identity* (%)	Base Pair	Accession	Samples origin
1	K1	*Anguilla bicolor bicolor*	98%	99.21%	643	KM875505.1	Kebumen
2	K2	*Anguilla bicolor bicolor*	87%	100%	644	MT107683.1	Kebumen
3	K6	*Anguilla bicolor bicolor*	99%	99.69%	651	MT107682.1	Kebumen
4	K8	*Anguilla bicolor bicolor*	98%	100%	651	KM875505.1	Kebumen
5	P6	*Anguilla bicolor bicolor*	97%	99.83%	615	KP979655.1	Purworejo
6	P8	*Anguilla bicolor bicolor*	96%	100%	649	KP979655.1	Purworejo
7	P9	*Anguilla bicolor bicolor*	98%	99.67%	609	KM875505.1	Purworejo
8	P10	*Anguilla bicolor bicolor*	99%	99.54%	650	MT107682.1	Purworejo
9	C2	*Anguilla bicolor bicolor*	98%	99.84%	644	KM875505.1	Cilacap
10	C4	*Anguilla bicolor bicolor*	98%	100%	640	KM875505.1	Cilacap
11	C6	*Anguilla bicolor bicolor*	90%	100%	644	MT107674.1	Cilacap
12	C7	*Anguilla bicolor bicolor*	99%	99.85%	650	MT107682.1	Cilacap

identified as *A. australis* from its morphology. There is a consensus between the two identification methods for samples C4, K1, and P6, which revealed the elvers species as *A. bicolor bicolor*.

Differences in morphological and molecular identification may occur because morphological assessment of the genus Anguilla is challenging due to species similarities, particularly in the young eel phase (elvers), as described in Tesch and Thorpe (2003) and Watanabe *et al.* (2008). For example, the morphology between *A. bicolor bicolor* and *A. bicolor pacifica* is very similar, where the value (D-A/PT)% and the total vertebrae are close to resemblance; 0.8 (D- A/PT)% with 109.5 spines for *A. bicolor bicolor* and 0.2 (D-A/PT)% with 107.1 spines for *A. bicolor pacifica*.

However, differences were found in this study, precisely in samples C7, K6, and P10 which were morphologically identified as *A. australis* with a (D-A/PT)% value close to 1.9 with smooth skin and parallelfin positions, as well as in samples P9, K2 and C2 where they were morphologically identified as *A. obscura* with values (D-A/PT)% close to 3.6 with smooth skin and parallel fins but molecular identification states as *A. bicolor bicolor* which should have morphological characteristics having values (D-A/PT)% close to 0.8, smooth skin and fin position parallel.

The sole variation between these three species is in the (D-A/PT) percentage. Given that the samples are elvers, this difference is considered to be caused by changes in the body form of species that have not yet evolved. Tesch and Thorpe (2003) supports this by stating that relatively few morphological features survive all stages before and after metamorphosis. Another notable distinction is the geographical distribution of *A. bicolor bicolor*, which is generally found

in Southeast Asia, while *A. australis* is mostly found on the Australian continent, and *A. obscura* is mostly found in Western Australia, Tahiti, and Samoa (Tesch and Thorpe, 2003).

This finding confirms the statement of Watanabe *et al*. (2004) that morphological characteristics alone are not sufficient to classify all species of this genus so that geographical distribution becomes an equally important characteristic. In addition, identification of eel species will also be very difficult to do if it is only based on morphology, especially at elver stage because of the similarity of morphological characters so molecular identification is necessary. It is because the differentiation of morphology on elver stage is an ongoing process, therefore it is difficult to identify morphologically. So morphological identification according to Tesch and Thorpe (2003) and Watanabe *et al*. (2008) based on the number of vertebrae have been used to help morphological identification in this study.

3.3 Phylogeny Analysis

The *A. bicolor bicolor* samples identified at the Jali River estuary in Purworejo have a very close relationship with *A. bicolor bicolor* samples found at the Luk Ulo Estuary of Kebumen and the Serayu River Estuary of Cilacap, according to the results of the phylogenetic tree (Figure 3). All of the samples obtained are also closely related, suggesting that the *A. bicolor bicolor* species samples found at the three research sites may be from the same evolutionary lineage. Furthermore, *A. bicolor bicolor* samples identified in Myanmar, India, Aceh, and Sukabumi have a tight association with *A. bicolor bicolor* found in Myanmar, India, Aceh, and Sukabumi. This is possible because the spawning locations of *A. bicolor bicolor* are in close proximity, supported by the statement of Arai (2016) which states that the spawning location of *A. bicolor bicolor* which occupies the Southeast Asian region is in the high seas of the western part of Sumatra Island which leads to the Indian Ocean. In addition, the very close genetic relationship between species is also caused by the migration pattern of most tropical eels which is different from eels in other climates, in other word the migration distance is much closer (Arai and Taha, 2021).

4. Conclusion

There are four species of *Anguilla* spp. identified morphologically in the estuaries of Jali River (Purworejo), Luk Ulo River (Kebumen), and Serayu River (Cilacap), namely *A. bicolor bicolor*, *A. bicolor pacifica*, *A. obscura*, and *A. australis*. Meanwhile, only one species was identified molecularly, i.e., *A. bicolor bicolor*. *A. bicolor bicolor* taken from the Jali River Estuary is closely related to those from the Luk Ulo River and the Serayu River. Furthermore, all samples are related to *A. bicolor bicolor* which is found in other parts of Indonesia and other countries where the tropical eels can be observed. When it is known that *Anguilla* spp. still has many mysteries such as migration patterns and spawning locations in Indonesian waters, this research is expected to provide information for further conservation research on the distribution of *Anguilla* spp. in Central Java.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow, Falah; collected samples, did research, drafted manuscript. Ratnawati; drafted manuscript. Adharini; lead and coordinated the research, drafted manuscript, and publication process. All authors; read and approved the final manuscript.

Conflict of Interest

The authors stated and declared that there is no conflict of interest in this research.

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