Taxonomic status investigation of freshwater eels (*Anguilla* spp.) based on the molecular marker in seven rivers that flow to Palabuhanratu Bay, Indonesia

A A Hakim,*, M M Kamal, N A Butet and R Affandi

Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, IPB University, Bogor 16680, ID

*Corresponding author: agusalimhakim0@gmail.com

Abstract. Freshwater eels (*Anguilla* spp.) are catadromous fish with widely distributed in the world and have long spawning migration. Palabuhanratu Bay is one of the potential areas and high activity for freshwater eels fishing. There have several rivers that flow to Palabuhanratu Bay. Species taxonomy certainty is needed to determine resource management. This study aimed to investigate the taxonomic status of *Anguilla* spp. based on Cytochrome Oxidase subunit I (COI) gene markers in seven rivers that flow to Palabuhanratu Bay. The sample was collected using timber traps (local name is *bubu*) from August and December 2014. The result of molecular identification obtained high agreement sequences that were morphologically identified six samples as *A. bicolor bicolor* and one sample as *A. marmorata*. Construction of the phylogeny tree indicated that intra-subspecies of *A. bicolor bicolor* had a close relationship and real apart from *A. marmorata* with a genetic distance range of 0.051–0.053. *A. bicolor bicolor* sequences of each river mixed and merged into one group. Molecular identification had shown taxonomy status of *Anguilla* spp. in several rivers that flow to Palabuhanratu Bay. Consequently, it could be determined for appropriate management.

1. Introduction

Freshwater eels (*Anguilla* spp.) are widely distributed in the world (tropical and subtropical) and have unique characters, such as a catadromous life history strategy, along with spawning migration, and a long leptocephalus larval period [1]. Nine species or subspecies of freshwater eels occur in Indonesia, i.e., *Anguilla bicolor bicolor* McClelland 1844, *A. nebulosa* McClelland 1844, *A. bicolor pacifica* (Schmidt 1928), *A. interioris* Whitely 1938, *A. borneensis* Popta 1924, *A. celebesensis* Kaup 1856, *A. marmorata* Quoy & Gaimard 1824, *A. obscura* Günther 1872, and *A. megastoma* Kaup 1856 [2]. Freshwater eels have distribution in Indonesia waters and known with certainties from Sumatera, Java, Kalimantan, Sulawesi, Bali, Nusa Tenggara, Maluku, and Papua [3]. There have been a few studies on tropical freshwater eels in Indonesia, i.e. migration [4–6], distribution [7, 8], species composition [4], recruitment [9], conservation genetic [3], diversity and abundance [10], and present and past genetic connectivity [11].

Palabuhanratu Bay, as one of the bay in the south of Java Island, is one of the potential areas and high activity for freshwater eels fishing. Sukabumi Regency has several rivers that flow to Palabuhanratu Bay, seven of them are Cibareno River, Cibangban River, Citiis River, Cisukawayana River, Citepus River, Cipalabuhan River, and Cimandiri River. Research about species composition of
freshwater eels in Palabuhanratu Bay based on morphology has done by Fahmi and Hirnawati [12] and Hakim [13]. Species taxonomy certainty is needed to determine the management of resources. Therefore, we need to investigate a molecular of Anguilla spp. from seven rivers that flow to Palabuhanratu Bay.

DNA barcoding is one of the molecular analysis techniques for identifying species. The DNA barcoding results are essentially composed of short DNA sequences from several individuals of a large number species [14], a short standardized sequence can distinguish individuals of a species by genetic variation [15]. Cytochrome oxidase subunit I (COI) in mitochondrial DNA can be used as molecular markers for species determination [15, 16]. COI has a rate of molecular evolution three times greater than 12S or 16S rDNA [17]. The evolution is rapid enough to allow discrimination not only closely allied species but also phytogeography groups within a single species [18, 19]. Previous molecular identification using the Cytochrome-b gene has been made on glass eels in the Cimandiri River [20]. This study aims to investigate taxonomic status from Anguilla spp. based on Cytochrome Oxidase subunit I (COI) gene markers on seven rivers that flow to Palabuhanratu Bay.

2. Materials and methods

2.1. Sample collection
The sample was collected using fish timber traps (local name is bubu) in seven rivers that flow to Palabuhanratu Bay, south of Java from August 2014 and December 2014. The research locations were Cibareno River, Cibangban River, Cititis River, Cisukawayana River, Citepus River, Cipalabuhan River, and Cimandiri River (Figure 1). The specimens were inserted into the collection tube with size 100 ml, contained 96% alcohol, and taken for analysis to the Laboratory of Aquatic Bimolecular, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University for molecular analysis.

![Figure 1. Sampling location in seven rivers that flow to Palabuhanratu Bay.](image)

2.2. Molecular analysis
DNA was isolated from tissue using a commercial kit (Gene Aid) based on factory manual protocol with some modifications. Total DNA will be generated in this stage, and the quality will be tested by electrophoresis on 1.2% agarose gel stained with Ethidium Bromide (EtBr). Electrophoresis used 100 mL of TAE1x buffer solution, 5 μL of ethidium bromide, and 2.5 μL of total DNA. Visualization of total DNA used ultraviolet machine.
Total DNA which has good quality was continued to amplifying DNA of fragments COI gene. Amplification was done by PCR (Polymerase Chain Reaction) using a commercial kit (Kapa Extra HotStart). Primers used a universal primer to some aquatic biota designed by Butet (2013 unpublished data). Stages amplification performed include pre-denaturation at 94°C for 5 minutes followed denaturation at 94°C for 45 seconds, annealing at 54°C for 1 minute, elongation at 72°C for 1 minute, post-elongation 72°C for 5 minutes, and storage of 15°C for 10 minutes. The quality of PCR products was tested by electrophoresis on a 1.2% agarose gel and visualized using an ultraviolet machine. PCR products have good quality continued to sequence step to determine the nucleotide sequence. PCR products were sent to sequencing services companies and were performed using the Sanger method [21].

2.3. Data analysis
The results of sequencing were aligned using the Clustal W method containing in the software MEGA 5.0 [22]. Nucleotide sequence of Anguilla spp. COI gene with forward and reverse primer was edited and analyzed to obtain the DNA sequences of the COI gene. The COI gene of this study was aligned among all samples and COI gene sequences for other species in the Anguilla genus found in GenBank. Data of COI gene sequences from other species were downloaded from GenBank. There are A. australis (EF609282.1), A. reinhardtii (HM006952.1), and A. japonica (HQ339972.1).

Genetic distances between Anguilla spp. from this study and other species of genus Anguilla from GenBank were calculated using pairwise distance method, were contained in the MEGA 5.0 program [22]. Genetic distance calculation results were presented in a matrix data that can be used for the analysis of kinship between species based on the phylogeny tree. Phylogeny analysis of Anguilla spp. was constructed between COI gene Anguilla spp. on this research with A. australis, A. reinhardtii, and A. japonica. Construction phylogeny tree used Neighbour-joining (NJ) bootstrapped method with 1000 repetitions containing in the MEGA 5.0 program [22].

3. Results
3.1. Morphological identification
Freshwater eels in Palabuhanratu Bay have obtained two species; there were Anguilla bicolor bicolor and A. marmorata. Freshwater eels with species of A. bicolor bicolor was obtained in each river, and A. marmorata was obtained in the Cibareno River. Measuring several morphometric characteristics was very useful to identify tropical eels in Palabuhanratu Bay. Species composition of freshwater eels was reported by Hakim [13]. We chose one individual from each river for molecular analysis. The sample code, species identification based on morphology, and sample origin were showed in Table 1.

3.2. Nucleotide sequence
COI gene amplification in each species of Anguilla has different targets. In this study, A. bicolor bicolor and A. marmorata were estimated at sites of 676 to 1,346 and 694 to 1,334. Both species refer to the complete COI gene of the same species; there were A. bicolor bicolor (AP007236.1) and A. marmorata (AP007242.1) (Figure 2).

Cytochrome oxidase subunit I (COI) gene nucleotide sequence was uploaded on BLASTn (Basic Local Alignment Search Tool-nucleotide) at the NCBI web site (National Center for Biotechnology Information). This gene was successfully amplified using PCR. The species identity verification of previously morphologically identified matches with the nucleotide sequence of identification based on mitochondrial cytochrome oxidase subunit I (COI) from the NCBI database (Table 2).

Cytochrome oxidase subunit I (COI) revealed definitive identity matches in 99% for all collected samples. Sample with code of ABBman1, ABBban1, ABBpal1, ABBsuk1, ABBti1, and ABBtep1 has proximity to A. bicolor bicolor (GenBank: AP007236.1) of 99%. AMbar1 have proximity to A. marmorata (GenBank: AP007242.1) of 99%. The E-value was 0.0 that showed highly significant similarities. It showed that ABBman1, ABBban1, ABBpal1, ABBsuk1, ABBtep2, and ABBti1 were
**A. bicolor bicolor**, while AMbar4 was *A. marmorata*. The NCBI reference sequences had high agreement with the samples that were morphologically identified six sample as *A. bicolor bicolor* and one samples as *A. marmorata*.

**Table 1.** Sample code, species identification based on morphology, and sample origin from each river.

| No. | Sample code | Species identification based on morphology | Sample origin (river)      |
|-----|-------------|---------------------------------------------|---------------------------|
| 1   | ABBman1     | *Anguilla bicolor bicolor*                  | Cimandiri                 |
| 2   | ABBban1     | *Anguilla bicolor bicolor*                  | Cibangban                 |
| 3   | ABBpal1     | *Anguilla bicolor bicolor*                  | Cipalabuhan               |
| 4   | ABBtii1     | *Anguilla bicolor bicolor*                  | Citiis                    |
| 5   | ABBsuk1     | *Anguilla bicolor bicolor*                  | Cisukawayana              |
| 6   | ABBtep1     | *Anguilla bicolor bicolor*                  | Citepus                   |
| 7   | AMbar1      | *Anguilla marmorata*                        | Cibareno                  |

**Figure 2.** The target gene of *A. bicolor bicolor* and *A. marmorata* based on COI complete gene of *A. bicolor bicolor* (AP007236.1) and *A. marmorata* (AP007242.1).

**Table 2.** Identification *Anguilla* spp. based on mitochondrial cytochrome oxidase subunit I (COI) sequences using BLASTn in Palabuhanratu Bay.

| Code   | Morphologically analyzed as | Species Identification | % Max identity | Accession     |
|--------|-----------------------------|------------------------|----------------|---------------|
| ABBman1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| ABBban1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| ABBpal1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| ABBtii1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| ABBsuk1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| ABBtep1| *A. bicolor bicolor*       | *A. bicolor bicolor*   | 99             | AP007236.1    |
| AMbar1 | *A. marmorata*             | *A. marmorata*         | 99             | AP007242.1    |

ABB = *Anguilla bicolor bicolor*, AM = *Anguilla marmorata*, bar = Cibareno River, ban = Cibangban River, tii = Citiis River, suk = Cisukawayana River, tep = Citepus River, pal = Cipalabuhan River, man = Cimandiri River.

The sequence of all samples was successfully submitted in the gene bank and became one of the genetic data of freshwater eel from Indonesia. The data can be accessed by researcher all-around of the world with the access code shown in Table 3.

This research found six specific nucleotide sites; *A. bicolor bicolor* and *A. marmorata* had 5 and 1 sites (Table 4). Those nucleotide sites were the specific genetic marker that can differentiate between two freshwater eel species.

Genetic distances between *Anguilla* spp. from this study and other species of genus *Anguilla* from GenBank were presented in a matrix data (Table 5). The genetic distance can be used for the analysis of kinship between species based on the phylogeny tree. In this study, the genetic distance of *A.*
bicolor bicolor has ranged from 0.00 to 0.016. Genetic distance between Anguilla spp. in this study with other species of genus Anguilla ranged from 0.051 to 0.076.

Table 3. Sample code, species identification based on morphology, and sample origin from each river.

| No. | Sample origin | Description | Accession |
|-----|---------------|-------------|-----------|
| 1   | Cimandiri     | Anguilla bicolor bicolor isolate ABBman1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY067460.1 |
| 2   | Cibangban     | Anguilla bicolor bicolor isolate ABBban1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765583.1 |
| 3   | Cipalabuhan   | Anguilla bicolor bicolor isolate ABBpal1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765585.1 |
| 4   | Citii         | Anguilla bicolor bicolor isolate ABBtii1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765584.1 |
| 5   | Cisukawayana  | Anguilla bicolor bicolor isolate ABBsuk1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765586.1 |
| 6   | Citepus       | Anguilla bicolor bicolor isolate ABBtep1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765587.1 |
| 7   | Cibareno      | Anguilla marmorata isolate AMbar1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial | KY765588.1 |

Table 4. Specific nucleotides of freshwater eel species.

| Species                        | Nucleotide position | 57 | 63 | 273 | 480 | 516 | 612 |
|--------------------------------|---------------------|----|----|-----|-----|-----|-----|
| A. bicolor bicolor Cimandiri   | A                   | A  | C  | G   | T   | A   |     |
| A. bicolor bicolor Cibangban   | A                   | A  | C  | G   | T   | A   |     |
| A. bicolor bicolor Citii       | G                   | T  | G  | A   |     |     |     |
| A. bicolor bicolor Cipalabuhan | A                   | A  | C  | G   | T   | A   |     |
| A. bicolor bicolor Cisukawayana| A                   | A  | C  | G   | T   | A   |     |
| A. bicolor bicolor Citepus     | A                   | A  | G  | G   | T   |     |     |
| A. marmorata Cibareno          | A                   | A  | C  | A   | T   | A   |     |
| A. marmorata (NC 006540.1)     | G                   | A  | C  | G   | T   | A   |     |

Table 5. Genetic distance matrix of COI gene fragment in A. bicolor bicolor, A. marmorata, A. australis, and A. reinhardtii based on pairwise distance method.

|              | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
|--------------|------|------|------|------|------|------|------|------|------|
| ABBman1      | 0.002|      |      |      |      |      |      |      |      |
| ABBban1      | 0.012| 0.011|      |      |      |      |      |      |      |
| ABBpall1     | 0.003| 0.002| 0.012|      |      |      |      |      |      |
| ABBsuk1      | 0.002| 0.000| 0.011| 0.002|      |      |      |      |      |
| ABBtep1      | 0.006| 0.005| 0.016| 0.003| 0.005|      |      |      |      |
| AMBar1       | 0.051| 0.053| 0.053| 0.051| 0.053| 0.051|      |      |      |
| A. marmorata | 0.051| 0.053| 0.053| 0.051| 0.053| 0.051| 0.003|      |      |
| A. australis | 0.070| 0.072| 0.073| 0.073| 0.072| 0.076| 0.066| 0.069|      |
| A. reinhardtii| 0.062| 0.061| 0.062| 0.062| 0.061| 0.062| 0.056| 0.059| 0.076|

Comparisons of A. bicolor bicolor samples with outgroup species obtained the lowest value of genetic distance with A. marmorata that is equal to 0.051. The highest genetic distance obtained between samples with A. australis at 0.076. Comparisons of A. marmorata samples with outgroup species obtained the lowest value of the genetic distance between samples with A. marmorata that is equal to 0.003. The highest genetic distance obtained sample with A. australis at 0.066.
Data from the genetic distance matrix used for the analysis of kinship was based on a phylogeny tree. Phylogeny tree *A. bicolor bicolor*, *A. marmorata*, *A. australis*, and *A. reinhardtii*, were constructed by pairwise genetic distance based on COI nucleotide (Figure 3).

Intra-subspecies of *A. bicolor bicolor* has a close relationship that shows in a contiguous tree branch. Construction of the phylogeny tree indicated that the specimens of *A. bicolor bicolor* real apart from the specimens of *A. marmorata* with a genetic distance value range of 0.051-0.053. Besides, samples of *Anguilla* spp. were a clear separation by outgroup with the value of the genetic distance of 0.056.

![Figure 3. Construction of phylogeny tree based on the COI gene in A. bicolor bicolor, A. marmorata, A. australis, and A. reinhardtii.](image)

4. Discussion

Indonesia was a potential country for freshwater eels as fisheries commodity to export market. Palabuhanratu Bay is one of the potential areas and high activity for freshwater eels fishing. The rivers that flow to Palabuhanratu Bay have found two species of adult phase and three species of glass eels in the Cimandiri River [12, 13]. Research about species composition of freshwater eels was only based on morphology characteristics. Fahmi et al. [23] has done molecular identification using semi-multiplex PCR that found three species of freshwater eels in Cimandiri River, i.e. *Anguilla bicolor bicolor*, *A. nebulosa nebulosa*, and *A. Marmorata*. The molecular identification of freshwater eels in other rivers has not been studied.

Molecular identification of *Anguilla* spp. using partial COI gene sequences have ascertained the truth and its proximity to other species based uploaded on BLAST-n (Basic Local Alignment Search Tool-nucleotide) at the NCBI web site (National Center for Biotechnology Information). The results of molecular identification showed that the sequences had high compatibility and supported the morphological identification in six samples as *A. bicolor bicolor* and one sample as *A. marmorata*. Sequences *A. bicolor bicolor* (GenBank: AP007236.1) and *A. marmorata* (GenBank: AP007242.1) were the research result of Minegishi [11]. *A. bicolor bicolor* samples obtained from Myanmar while *A. marmorata* samples obtained from Ambon, Indonesia. Six samples of *Anguilla* spp. have closed to *A. bicolor bicolor* by 99%. One sample *Anguilla* spp. has closed to *A. marmorata* by 99%. Differences in genetic distance between *Anguilla* spp. with other species obtained from GenBank were greater than 3% indicate that certain different molecular species present in GenBank [15]. The results obtained showed that proximity is very high at 99%, so the truth could be ascertained that there were two species, namely *A. bicolor bicolor* and *A. marmorata*.

Certainty taxonomy is needed in determining resource management, especially on aquatic biota recurring phenomena of cryptic species and complex species [24, 25]. A taxonomic species that have morphologically obvious needs to identify a molecular basis for determining nucleotide sequences. The results of nucleotide sequences can be used to determine species evolution. Molecular identification of this study has shown taxonomy certainty of *Anguilla* spp. in several rivers that flow to Palabuhanratu Bay so that it can be determined for appropriate management.
Comparisons of *A. bicolor bicolor* samples in different locations obtained value of genetic distance with range 0.001-0.016. *A. bicolor bicolor* sequences of each river mixed and merged into one group. The presence of *A. bicolor bicolor* in the south of Java was thought to have spawning areas in the Mentawai waters [26]. Larvae carried away by turbulence and flowed towards the coast, started to go into the river and estuarine waters when it has been turned into glass eel stadia. Allegedly there was connectivity on each river because they have the same location of spawning.

The results of the nucleotide base from *A. bicolor bicolor* of this study can be compared with other studies in different locations. The different sequences of nucleotide bases (insertions) and missing (deletions) can be used to know origin species or species distribution. The decline in genetic diversity can occur naturally through drift genes. Random genetic drift illustrated the change randomly within a population which resulted in reduced genetic diversity from generation to generation that was not caused by the pressure of the environment, but because of a mutation in a pure [27]. Besides, genetic information can be used as a basis for determining population genetics and stock assessment of freshwater eels. If an area has a low genetic diversity of freshwater eels due to the pressure of inbreeding and outbreeding, it is necessary to the conservation of species.

5. Conclusions

The result of molecular identification showed sequences had a high agreement that was morphologically identified six samples as *A. bicolor bicolor* and one sample as *A. marmorata*. The sequence of all samples had available in the gen bank. Construction of phylogeny tree indicated that intra-subspecies of *A. bicolor bicolor* has a close relationship and *A. bicolor bicolor* real apart from *A. marmorata* with a genetic distance value range of 0.051-0.053. *A. bicolor bicolor* sequences of each river mixed and merged into one group. Molecular identification has shown taxonomy certainty of *Anguilla* spp. in several rivers that flow to Palabuhanratu Bay so that it can be determined for appropriate management.

Acknowledgments

We would like to express our thanks to the Ministry of Research, Technology, and Higher Education who gave funding for this research. The author also expresses his gratitude to all those who helped complete the research.

References

[1] Watanabe S 2003 *Taxonomy of the freshwater eels, Genus Anguilla Schrank*, 1798 ed K Aida, K Tsukamoto and K Yamauchi (Tokyo: Springer) pp 3-18
[2] Sugeha H Y and Suharti S R 2008 Discrimination and distribution of two tropical short-finned eels (*Anguilla bicolor bicolor* and *Anguilla bicolor pacifica*) in the Indonesia waters *The Nagisa Westpac Congress* 9 1-14
[3] Fahmi M R 2015 Short communication: Conservation genetic of tropical eel in Indonesian waters based on population genetic study *Proceeding Seminar Nasional Masyarakat Biodiversitas Indonesia* 1(1) 38-43
[4] Arai T, Aoyama J, Limbong D and Tsukamoto K 1999 Species composition and inshore migration of the tropical eels *Anguilla* spp. recruiting to the estuary of the Poigar River, Sulawesi Island *Marine Ecology Progress Series* 188 299-303
[5] Aoyama J, Wouthuyzen S, Miller M J, Inagaki T and Tsukamoto K 2003 Short-distance spawning migration of tropical freshwater eels *The Biological Bulletin* 204 104-108
[6] Sugeha H Y, Aoyama J and Tsukamoto K 2006 Downstream migration of tropical anguilid silver eels from lake Poso, Central Sulawesi, Indonesia *Limnотek* 8(1) 18-25
[7] Aoyama J, Wouthuyzen S, Miller M J, Minegishi Y, Kuroki M, Suharti S R, Kawakami T, Sumardiharga K O and Tsukamoto K 2007 Distribution of leptocephali of the freshwater eels, genus *Anguilla*, in the waters off west Sumatra in the Indian Ocean *Environmental Biology of Fishes* 80 445-452
[8] Sugeha H Y and Suharti S R 2008 Discrimination and distribution of two tropical short-finned eels (*Anguilla bicolor bicolor* and *Anguilla bicolor pacifica*) in the Indonesia waters *The Nagisa Westpac Congress* 9 1-14

[9] Sugeha H Y, Arai T, Miller M J, Limbong D and Tsukamoto K 2001 Inshore migration of the tropical eels *Anguilla* spp. recruiting to the Poigar River estuary on north Sulawesi Island *Marine Ecology Progress Series* 221 233-243

[10] Miller M J, Wouthuyzen S, Ma T, Aoyama J, Suhartib S R, Minegishi Y and Tsukamoto K 2011 Distribution, diversity, and abundance of garden eel larvae off West Sumatra, Indonesia *Zoological Studies* 50(2) 177-191

[11] Minegishi Y, Gagnaire P A, Aoyama J, Bose P, Feunteun E, Tsukamoto K and Berrebi P 2011 Present and past genetic connectivity of the Indo-Pacific tropical eel *Anguilla bicolor* *Journal of Biogeography* 1-13

[12] Fahmi M R and Hirnawati 2010 Keragaman ikan sidat tropis (*Anguilla* sp.) di perairan Sungai Cimandiri, Pelabuhan Ratu, Sukabumi *Prosiding Forum Inovasi Teknologi Akuakultur* 1-8

[13] Hakim A A, Kamal M M, Butet N A and Affandi R 2015 Komposisi species ikan sidat (*Anguilla* spp.) di delapan sungai yang bermuara ke teluk Palabuhanratu, Sukabumi, Indonesia *Jurnal Ilmu dan Teknologi Kelautan Tropis* 7(2) 573-585

[14] Hajibabaei M, Singer G A C, Hebert P D N and Hickey D A 2007 DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics *TRENDS in Genetics* 23(4) 167-172

[15] Hebert P D N, Ratnasingham S and De Waard J R 2003 Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species *Proceedings of the Royal Society of London B* 270 96–99

[16] Solihin D D 1994 Peran DNA mitokondria (mtDNA) dalam studi keragaman genetik dan biologi populasi pada hewan *Hayati* 1(1) 1-4

[17] Knowlton N and Weigt LA 1998 New dates and new rates for divergence across the Isthmus of Panama *Proceedings of the Royal Society of London B* 265 2257-2263

[18] Cox A J and Hebert P D N 2001 Colonization, extinction and phylogeographic patterning in a freshwater crustacean *Molecular Ecology* 10 371-386

[19] Wares J P and Cunningham C W 2001 Phylogeography and historical ecology of the North Atlantic intertidal *Elise* 12 2455-2469

[20] Fahmi M R 2013 *Phylogeography of tropical eels (Anguilla spp) in Indonesia waters (Thesis)* (Bogor: Bogor Agricultural niversity)

[21] Sanger F, Nicklen S and Coulson A R 1977 DNA sequencing with chainterminating inhibitors *Proceedings of the National Academy of Sciences USA* 74 5463-5467

[22] Tamura K, Dudley J, Nei M and Kumar S 2011 Mega 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods *Molecular Biology and Evolution* 28(10) 2731-2739

[23] Fahmi M R, Solihin D D, Soewardi K, Pouyaud L, Shao Z and Berrebi P 2013 A novel semimultiplex PCR assay for identification of tropical eels of genus *Anguilla* in Indonesian waters *Fisheries Science* 79 185-191

[24] Bickford D, Lohman D J, Sodhi N S, Ng P K L, Meler R, Winker K, Ingram K K and Das I 2006 Cryptic species as a window on diversity and conservation *Ecology and Evolution* 22 148-155

[25] Arlyza I S, Shen K N, Solihin D D, Soedharma D, Berrebi P and Borsa P 2013 Species boundaries in the *Himantura uarnak* species complex (Myliobatiformes: Dasyatidae) *Molecular Phylogenetics and Evolution* 66 429-435

[26] Arai T 2014 Do we protect freshwater eels or do we drive them to extinction? *SpringerPlus* 3 534

[27] Singleton P and Sainsbury D 2006 *Dictionary of Microbiology and Molecular Biology, Third Edition* (England: Wiley & Sons Ltd) p 889