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DNA BARCODING AND PHYLOGENIC ANALYSIS OF TROPICAL EELS (*ANGUILLA* SPP.) BASED ON PARTIAL D-LOOP AND CYT B GENES IN THE NORTH MALUKU PACIFIC WATERS

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Abstract

The North Maluku waters is one of tropical waters, place of distribution and spread of anguillid eels (Anguilla spp.) in Indonesia. The structure of molecule of eel species and composition of its population inhabiting the waters is important to be observed to determine the management of supply of eel species seedlings. The aim of this study is to do molecular identification, analyse the genetic distance and phylogenic tree of eel based upon the partial Displacement Loop and Cytochrom b genes as the genetic marker. This study used 14 eel samples collected from 3 locations in North Maluku (Bacan, Tidore and West Gane). Partial D-Loop and Cyt b genes were amplified using the primary partial D-Loop and Cyt-b genes and then the similarities of the sequencing results were analyzed using Blastn NCBI. The analysis of genetic distance and phylogenic tree were conducted by means of Kimura-2-Parameter method using MEGA X (Molecular Evolutionary Genetic Analysis) program. The results showed two types of anguillid species identified: Anguilla marmorata (97-100%) and Anguilla interioris (100%). Anguilla marmorata is a species with high spreading rate, and it can be found in all locations. Meanwhile, Anguilla interioris is the species with limited spreading and it can be only found in West Gane. The average of genetic distance between populations had a medium value (0.33), indicating that there was a variety of genetics. Based upon the analysis of phylogenic tree, Anguilla marmorata spread in North Maluku is considered to come from the different breeds but has mixed in nature in view of migration process.

Keywords*: Distribution; Genetic connectivity; Pacific biodiversity; Tropical eel; Tropical biodiversity*

Introduction

Anguillid Eels (*Anguilla* spp.: Anguillidae) have a unique life cycle having catadromous migration in which eels start to live at sea, migrate to freshwater to grow into adults and return to the sea for spawning [1]. In Indonesia, anguillid eels become one of quite important fisheries commodities for international market trade. Indonesia becomes the largest exporter of eels in which the number of annual exports can reach thousand tons with the amount of million dollars. The eel is also one of favourite food in Japan as well as in a number of countries and continents such as Europe, America, Taiwan, South Korea, and Middle East.

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The increasing demand for eels worldwide in every year is related to its nutrient content, benefits for health and being the favourite food in some countries [2].

Information about the species diversity in each region in Indonesia is deemed important as a base to determine the managerial policies and conservation. The diversity between the species of eel can be seen from a number of characters including the comparison between preanal and predorsal length (as a specific character), shape of head, number of miomers, teeth structure, and number of backbones [3]. Nevertheless, the difference in morphological characters can only be used to differentiate eel in the adult size and living in a similar stadium. Therefore, it requires a molecular approach to validate such difference such as in the use of genetic markers to identify the species of eel [4, 5]. The genetic identification is not only for determining the species of fish but also for determining the diversity of genetics between species and for tracking the origins of ancestors or genetic relationship of eel fish through the phylogenic tree analysis.

North Maluku is one of the areas in Indonesia for anguillid eels spreading and has a great potency in providing the seedlings of eels. A number of studies on tropical anguillid eels have been reported [6-12], however, the previous studies have not given the information on the species of eel genetically in North Maluku area. The studies about the species of eel and information of the population genetically have never been conducted particularly in North Maluku waters. Morphologically similar specimens in eels making it difficult in identification. The almost similar key morphological characteristics has made it difficult to differentiate any specimen morphologically. It then needs the DNA barcoding method to make an accurate identification at the species level [13]. The study of organic outer shape (morphology) can be used as a key for the identification of population structure, but it does not provide any genetic information [14].

The morphological identification to the molecular identification was based on the short DNA segment called as DNA *Barcodes* [15]. DNA *barcoding* is a favourite method in taxonomy forensic for being effective in identifying the test sample and does not result in the valid data [16, 17]. Obtaining genetic information is important aimed to preserve the endangered species; thus, conservation efforts are deemed critical [18, 19]. This study firstly provides species diversity and composition by means of DNA barcoding in North Maluku. The information can be used as the recommendation in making the policies about the optimization of the conservation of eel.

Experimental

Materials

This research was conducted from June 2018 to December 2019. The sampling of eels was conducted in 3 locations: Tidore, Bacan and West Gane (North Maluku, Indonesia) (Fig. 1). Sample collection was carried out at the river mouth using a net. After collection, morphological characteristics such as total length, head length, standard length, body height, head height, fin length, height of tail and body circumference were measured. The tissue samples were dissected and then sent to the Laboratory of BIONESIA (Biodiversitas Indonesia) in Bali for genetic analyses such as DNA isolation and amplification of partial D-Loop and Cyt b genes. The tools used for the extraction included 1.5mL tube, a set of micro-pipettes along with its tape, vortex, centrifuge, freezer, and incubator. Tools used for amplification and electrophoresis included the PCR *thermocycler* machine, vortex, micro *centrifuge*, PCR tube, refrigerator, a set of gel maker tray, digital scale, microwave, stirrer, 100v power supply, and UV Transilluminator. The materials used included TNES (Tris-base 10mM, NaCl 125mM, EDTA 10mM pH = 8, SDS 0.5%) solution, PCIAA (Phenol Chloroform Isoamil Alchohol), TE

(Tris EDTA), Mix PCR, Primer forward and reverse gen CYTB, Agarose Gel, buffer TBE, and florosave.

Methods

DNA extraction in this research used the TNES method. The first phase in DNA extraction was by the destruction of membranes and cell walls using TNES buffer. Cell splitting (lysis) is the initial stage of DNA isolation aimed to release the contents of cells. The TNES buffer contents included Tris-HCl, NaCl, EDTA and SDS. NaCl solution acts to stabilize the solution to accelerate the reactions that will occur in the next phase. EDTA (ethylenediaminetetraacetic acid) is the inhibitor compound that can inhibit the activities of nuclease enzymes that will form complexes (chelates) with metal ions, such as Mg^{2+} as a DNAse cofactor. SDS (Sodium Dodecyl Sulphate) solution acts to destroy the lipid in the cell membranes. The process can cause the degradation to DNA destruction due to the activities of endonuclease enzymes; therefore, it needs to use extraction buffer containing Tris compounds.

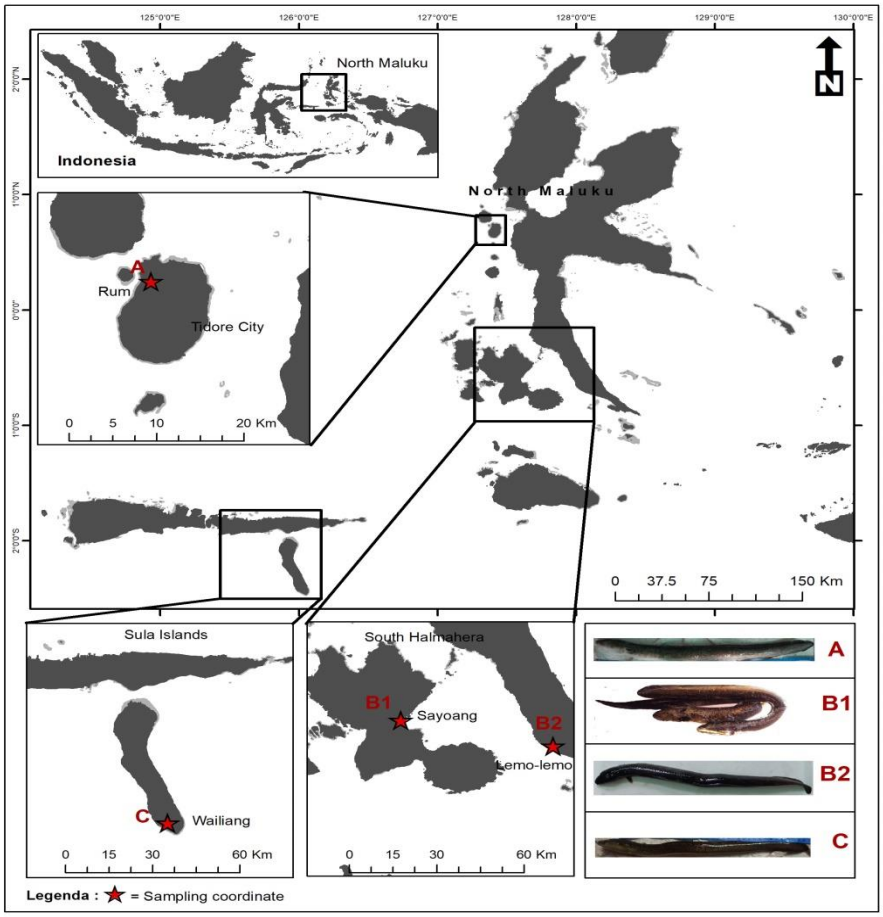

Fig 1. The Map of Sampling Location in North Maluku – Indonesia:
a. Tidore; b1. Bacan ; b2. West Gane

The following process was the separation of DNA from the cell components or contaminants undesired. This separation of DNA from other cell components including cell debris was conducted using centrifugation. Supernatants containing DNA were placed on different containers. To maximize the results of isolation, it was then added with phenol into the

supernatant. Phenol is an organic solvent that acts to dissolve proteins, lipids, and other molecules such as polysaccharides with an expectation to obtain a supernatant containing the contaminant-free DNA. The centrifugation was again conducted to the mix to separate the DNA from undesired contaminants. The addition of absolute ethanol was used as the agent of DNA precipitation. As its name implies, precipitation aimed to precipitate histone proteins, so that DNA strands no longer curl and bind to histone proteins, which causes DNA to be visible. This can be seen through the presence of DNA deposition threads on the bottom of the tube. The appearing DNA strands were carefully removed and transferred into the microtube. Furthermore, alcohol still wetting the DNA was evaporated in a vacuum to make DNA dried and ready for further testing.

The amplification of partial D-Loop and Cyt b genes was conducted using PCR (*Polymerase Chain Reaction*) method, a method to double the DNA sequence in a short time in vitro. Denaturation at 95°C was held for 3 seconds, annealing (Ta) at 52°C was held for 30 seconds, Elongation at 72°C was held for 1 minute and going to step 2 (30× cycles). The final extending at 72°C was held for 5 minutes. The reaction of amplification of DNA fragment was started with denaturation of DNA template to separate the *double stranded* to be *single stranded*. It was continued with the *annealing phase* that is the attachment of primary sequences to the single-stranded DNA template complementarily. Once finishing annealing, it was continued with the polymerization process (*elongation*) of the new DNA chain based upon the DNA template.

The achievement of DNA amplification was then measured qualitatively by means of the electrophoresis method, by separating a molecule through agarose gel under the electric field state. Negatively charged DNA with negative charge moved towards the positive pole. The speed of movement was affected by the size of the molecular weight. Good DNA appeared as a single band without impurities, and it was clearly visible under UV light. The partial D-loop and Cyt b gene amplifiers of the fourteen samples were sequenced, and then they were aligned with the BLASTn program (*Basic Local Alignment Search Tool nucleotide*) to find a database that had a high similarity to the sample. *Multiple Sequence Alignment* was then conducted for further analysis on the diversity of samples and phylogenetic.

Results and discussion

Morphology characteristics

The external morphology showed the total length of fifteen samples in four locations (Table 1). The longest sample was found in 80.2cm from sample S3 from Bacan (BCN) and the lowest on was in 27cm from sample S5 from Tidore (TDR).

Table 1. Morphology of Eel Samples in Four (4) Locations

Morphometric	TDR					BCN				West Gane				
(cm)	S1	S2	S3	S4	S5	S1	S2	S3	S4	S1	S2	S3	S4	S5
Total Length	62	56	52	40	27	40	47.5	80.2	79.2	54	43	30	51	46
Head Length	10	9	8	7	4	7	8	12	11.5	7.8	6.8	4,7	8	7.1
Standard Length	57	52	48	37	23	37	5	77.7	76.8	48.6	39	25	47	42
Body Height	7	6	5	4	3	2.7	3,5	6.7	6.2	4.3	5	3.3	4.9	5.4
Head Height	6	6	4	4	3	2.5	3	9	8.6	4	4.2	4	4	4.8
Fin Length	3	4	3	2	2	2	2,3	5.6	5.1	3.4	2.3	2.5	3	2.7
Height of Tail	5	5	4.5	4	3	2.5	3.3	5.2	4.9	4.6	4,2	3.3	4.3	4.6
Body Circumference	14	13	11	9	7	8	10	19	18	11.3	9.8	7.8	10.8	10

*remark: TDR = Tidore, BCN = Bacan, GB = West Gane

The longest size of head was at 12.2cm from sample S3 from Bacan and the lowest one was at 4cm from sample S5 from Tidore. The standard length was 77.2cm from sample S3 from Bacan and the lowest one was at 23cm from sample S5 from Tidore. The size of body height was at 6.2cm from sample S3 from Bacan and the lowest one was at 3cm from sample S5 from Tidore. For the height of head was at 9cm from sample S3 from Bacan and the lowest one was at 2.5cm from sample S5 from Tidore. The length of fin was at 5.6cm from sample S3 from Bacan and the lowest one was at 2cm from sample S5 from Tidore and the height on tail was 3cm from sample S3 from Bacan and the lowest one was at 2cm from sample S5 from Tidore. Meanwhile, the highest body circumference was at 19cm from sample S3 from Bacan and the lowest one was at 7cm from sample S5 from Tidore.

DNA Barcoding

Mitochondria DNA (mt DNA) has a number of typical genetic characteristics that are different from core genome. Mt DNA is only passed down through the mother's path without recombination [20]. Such unique heredity system has been used in any fields such as to determine the kinship, study on evolution, migration, and identification of genetic disease [21]. Cytochrome b (Cyt b) mitochondria gene has been widely used in study and resulted in a difference in any taxonomy levels. In the study of [22], the use Cyt b as the molecular marker has been tested in determining the phylogenic relation in any taxa levels in Cichlidae family.

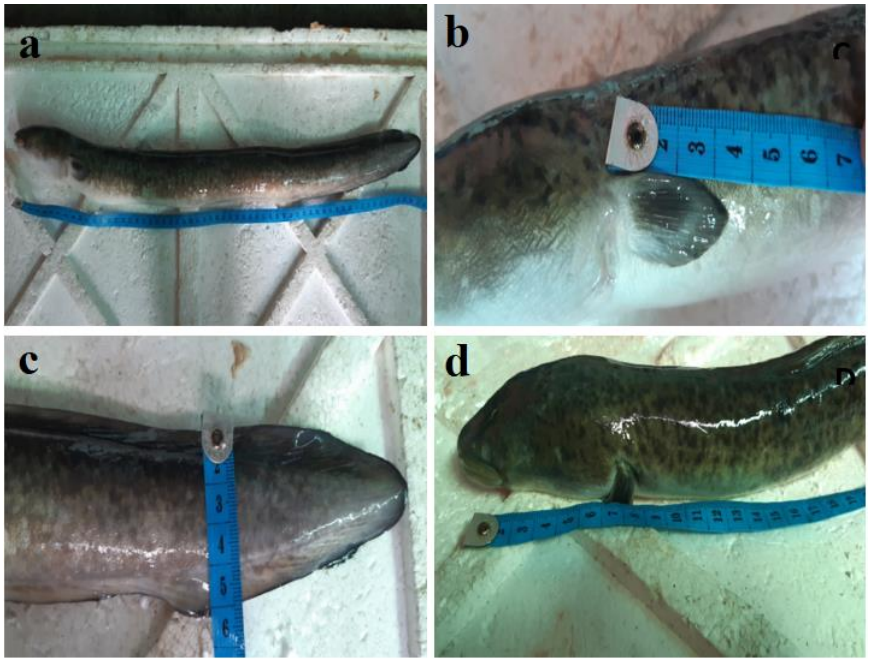

Fig. 2. Measurement of Eel Morphology: a. Total Length; b. Height of Tail; c. Length of Fin; d. Head Length.

Displaced Loop gene, also called as mitochondria control area, is a non-coding area that has a high polymorphism level. The analysis of variations in the D-Loop nucleotide sequence can be used to determine the identity of individual and maternal kinship relationship. The amplification of partial D-Loop and Cyt b genes is highly determined by the condition of primary attachment on the sample of genom DNA. The results of the gene amplification showed the varied number of alkali-rich fragments in the range of 536-584bp. The variation in the number of alkali-rich fragments was related to the insertion or deletion of nucleotide alkali

in the targeted gene areas. The partial D-Loop and Cyt b genes from 14 samples of DNA of eel (*Anguilla* sp.) were successfully amplified and resulted in the amplicon sized 536-584bp. Table 2 ~~below~~ presents the identification based upon the gene similarities towards the database using BLASTn program that can be accessed through NCBI site.

The result of identification based upon the similarities of sample of eel in Maluku waters showed the compatibility with the species *Anguilla marmorata* with the similarity level of 97-100% and *Anguilla interioris* with the similarity level of 100%. The result of the identification showed that the spread of eel has been dominated by *Anguilla marmorata*, found in each sampling location. Whereas *Anguilla interioris* was only found in one location: West Gane. This is in line with the statement of mentioning [1], that some of eel species in Indonesia have been widely distributed and others are distributed limitedly. *A. celebesensis*, *A. borneensis* and *A. interioris* are distribute in Indonesian waters [3]. These three species are the endemic species in Indonesia waters, while *A. marmorata* and *A. bicolor* are widely spread.

The sequence of sample was aligned with *Multiple Sequence Alignment* using Clustal O Program. The genetic distance between samples was then calculated to describe the differences of nucleotide alkali between population and diversity among species. The genetic distance (d) between samples of eel in North Maluku waters were analyzed using *Pairwise Distance* of Kimura 2-Parameter model using MEGA X (*Molecular Evolutionary Genetic Analysis*) program (Table 3).

Samples were grouped into 3 based upon the location of sampling: population of Bacan, Tidore, and West Gane. The averages of genetic distance (d) in population and among population were analysed using Kimura 2-Parameter model [23] (Table 3 and Table 4). Genetic distance is a measure of genetic divergence between species or between populations within a species either in the distance of measuring the time of the same ancestors or the level of differentiation [24]. The estimated value of genetic distance (d) of eel samples in the population of Bacan and West Gane showed a higher value compared to the one in Tidore population(Table 5)The high value of genetic distance indicated that the population originated from the mixing of different ancestors. Tidore geographically is the farthest from the population of Bacan and West Gane; it has a genetic distance close to 0.

Table 2. Identification of Eel (*Anguilla* sp.) sample in North Maluku, based upon similarities of partial D-Loop and Cyt B genes, using the Database of Reference from NCBI

Sample	Alkali Length (bp)	Similarities (%)	Reference Species	Acc. Number
Bcn1	536	100	*Anguilla marmorata*	HQ388831.1
Bcn2	574	100	*Anguilla marmorata*	HQ388830.1
Bcn3	574	99	*Anguilla marmorata*	HQ388830.1
Bcn4	575	99	*Anguilla marmorata*	HQ388831.1
Tdr1	574	100	*Anguilla marmorata*	HQ388830.1
Tdr2	574	100	*Anguilla marmorata*	HQ388830.1
Tdr3	575	100	*Anguilla marmorata*	HQ388826.1
Tdr4	574	99	*Anguilla marmorata*	HQ388830.1
Tdr5	576	99	*Anguilla marmorata*	HQ388825.1
GnBr1	575	100	*Anguilla interiors*	AP007241.1
GnBr2	578	100	*Anguilla marmorata*	HM802168.1
GnBr3	581	97	*Anguilla marmorata*	HM802174.1
GnBr4	584	99	*Anguilla marmorata*	HM802170.1
GnBr5	577	100	*Anguilla marmorata*	HQ388825.1

*remark: Bcn = Bacan; Tdr = Tidore; GnBr = West Gane

The common size of genetic distance used is the fixation index in variation of 0 and 1. The value of 0.03 in the population of Tidore showed that the population is identical genetically (the genetic variety is minimum or none); meanwhile, value 1 showed the genetic difference in

the population (maximum diversity). This also can be seen from the analysis on the similarities in Tidore population in which there is only one *Anguilla marmorata* identified. The average of genetic distance of eels of 0.33 in North Maluku showed that the genetic difference is at moderate level in the region. The estimation of genetic distance between populations analyzed to understand the biodiversity level. As shown in Table 4, it can be found that the population in West Gane–Bacan have a higher genetic distance than that of Bacan-Tidore and West Gane - Tidore. Though West Gane-Bacan geographically have the closest distance (±72.6km) in comparison to Bacan-Tidore (±136km) and West Gane-Tidore (±170km), the genetic distance of the population showed a higher difference. This then showed that the population in West Gane-Bacan have a quite high diversity. The genetic difference occurred between the population can be determined by the spread of larvae due to the oceanographic factor causing the formation of sub-population [25, 26].

Table 3. The estimation of genetic distance (d) among the sequences of Cyt b genes of eel *Pairwise Distance* of Kimura 2-Parameter model [23]

SAMPEL	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	1.00													
3	1.05	0.02												
4	1.02	0.03	0.02											
5	1.05	0.02	0.03	0.04										
6	1.05	0.02	0.00	0.02	0.03									
7	1.01	0.03	0.02	0.01	0.03	0.02								
8	1.01	0.03	0.03	0.04	0.04	0.03	0.03							
9	1.02	0.03	0.02	0.01	0.04	0.02	0.01	0.03						
10	1.19	0.24	0.24	0.24	0.23	0.23	0.23	0.24	0.24					
11	1.06	0.07	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.26				
12	0.07	1.03	1.06	1.04	1.07	1.07	1.04	1.03	1.04	1.21	1.05			
13	1.04	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.25	0.02	1.02		
14	1.02	0.04	0.03	0.02	0.05	0.03	0.02	0.05	0.01	0.24	0.08	1.05	0.08	

Legend: 1. Bacan1; 2. Bacan2; 3. Bacan3; 4. Bacan4; 5. Tidore1; 6. Tidore2; 7. Tidore3; 8. Tidore4; 9. Tidore5; 10. West Gane1; 11.West Gane2; 12.West Gane3; 13.West Gane4; 14.West Gane1

Table 4. The estimation of the Average of Genetic Distance (d) of sample of eel in population using Kimura 2-Parameter Model [23]

Population	n	D
Bacan	4	0.52
Tidore	5	0.03
West Gane	5	0.52
Total Population	14	0.33

Table 5. The estimation of Genetic Distance (d) between populations of eel using Kimura 2-Parameter Model [23]

Locations	Bacan	Tidore	West Gane
Bacan	-	-	-
Tidore	0.273	-	-
West Gane	0.435	0.290	-

Biologically, the living phase of marine organisms starts from planktonics in the water column. The planktonic phase allows the organism to be carried away by the flows and then separated from the original parent group (lineage). *T. Arai et al* [9] Stated that the eel larvae

experiences the development to be juvenile (glass eel) with a shape that resembles the anguilid and transparent pipe. Eel Juveniles then are carried away by the tide to the river mouth and live several days in the estuary to adapt to changes in salinity [5]. *B. Gaylord and S.D. Gaines* [27] stated that the sea wave can affect the distribution of population distribution and structure of fish genetics. *A.L. Gordon and R.A. Fine* [28] revealed that the process of the changes of genes occurs between populations in tropical areas in Indo-Pacific causing the genetic proximity between populations.

Phylogenic

The identification of eel was also conducted using the phylogenic tree approach to determine the kinship and the history of eel evolution in the North Maluku waters with a number of other species (Fig. 3). The method used to reconstruct the phylogenic tree was *Neighbour-Joining* method using MEGA X program. This method is more widely used to obtain the accurate phylogenic relationship coming from DNA sequence. Three of fourteen sequence samples that had different similarity values were chosen to represent the samples of eel from North Maluku through the analysis of phylogenic tree. In the analysis, the sample of eels from Maluku was aligned with a number of sequence of eel references that have ever been found in Indonesia, including *Anguilla marmorata (*AB279061.1; HM802168.1; HQ388826.1), *Anguilla interioris* (NC_006539.1; AP007241.1; MG977381.1), *Anguilla celebesensis (*NC_006537.1), *Anguilla nebulosa nebulosa (*NC_006544.1), *Anguilla bicolor bicolor (*NC_006534.1), *Anguilla bicolor pacifica* (NC_006535.3) and *Anguilla rostrata (*NC_006547.2)

Based on the analysis of phylogenic tree, it has been found that partial D-Loop and Cyt b genes from the sample of eel of North Maluku were in the different cluster. The sample of Bacan-1 was in the similar cluster with the isolate *Anguilla marmorata* (Acc.no: HQ388831.1) with the level of similarities of 100%. Though being identified as the similar species that is *Anguilla marmorata*, the sample of Tidore-3 in the phylogenic tree was separated in another cluster. The sample had the similarities 100% with the isolate *Anguilla marmorata* (HQ388826.1). Meanwhile, in sample West Gane-1, the position in the phylogenic tree is in the similar cluster with the isolate *Anguilla interioris* (AP007241.1) with the level of similarities of 100%. The difference of cluster showed the difference of *ancestor* from the tree populations of eel in North Maluku area. This is strengthened with the values of genetic distance showing a genetic difference from the similar species of eel.

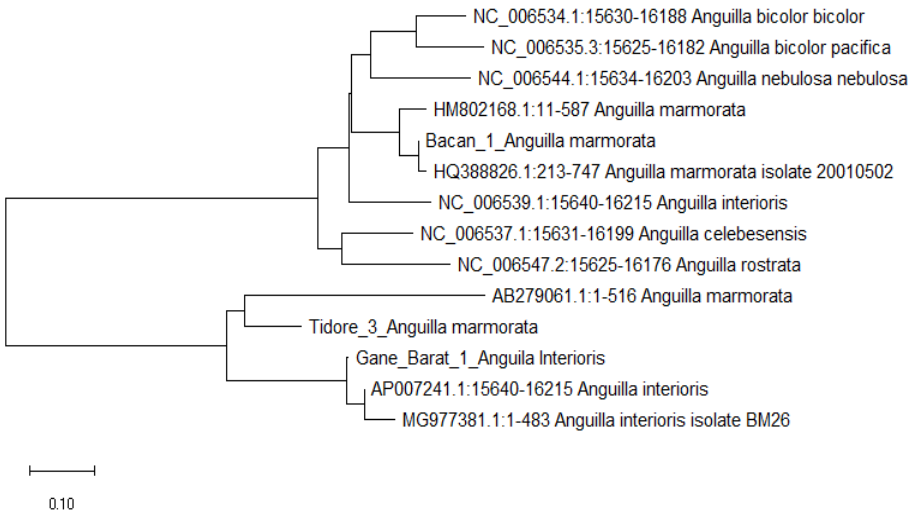

Fig. 3. Phylogenetic tree Eels (*Anguilla* sp.) in North Maluku and haplotypes reference from NCBI *GeneBank* based on gen D-Loop dan Cyt b partial using *Neighbour-Joining*

As stated in references [29-36], genetic conservation is oriented to the optimization of genetic potential, minimization of inbreeding potential of a species, fishery management and conservation. This is purposely to make the genetic diversity of a species can be a base in the management or conservation of genetics. The species that have low genetic diversity need to be given protection. Meanwhile, the utilization is prioritized to the species that have high diversity. In this research, it was found that *Anguilla interioris* is an endemic species and has a narrow distribution. This species additionally has a small population*;* this certainly needs to maintain its sustainability through protection and the limitation in its use. Nevertheless, this becomes a certain constraint in the implementation as *Anguilla interioris* morphologically has similarity with *Anguilla marmorata* that has high population and wide distribution [1].

Based on the analysis on partial D-Loop and Cyt b genes, fourteen samples of eel in North Maluku were identified as *Anguilla marmorata* (13 samples with similarities of 97-100%) and *Anguilla interioris* (one sample with similarities of 100%). The genetic distance of eel (*Anguilla sp.*) in North Maluku waters was categorized as medium (average of d = 0,33), the genetic distance showed the genetic difference in eel population. Based on the phylogenic tree, *Anguilla marmorata* distributes in North Maluku is considered to come from a different ancestor but they have experienced a mixture in nature.

Conclusion

Our research is a preliminary study that indicates individuals based on morphological characteristics that are examined using DNA Barcoding. It has been found that the identified species was Anguilla. Genetic distance represents a genetic limitation, so initial conclusion is that there are distinct groups. Molecular DNA has shown that can describe morphology based on the sequence of DNA sequences, thus facilitating and speeding up the identification process. This research is very important in the future of population studies.

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