DNA Barcoding of Anchovy in Tuban Regency as Database of Indonesian Marine Genetic Diversity

Marita Ika Joesidawati[1]*, Nining Nursalim[2], Nenik Kholilah[2], Eka Maya Kurniasih[3], Ni Kadek Dita Cahyani[2,4], Ambariyanto Ambariyanto[2,5]

[1]*Study Program of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas PGRI Ronggolawe Tuban*
Jl. Manunggal 61 Tuban, East Java, 62381, Indonesia
[2]*Diponegoro Marine Biodiversity Project Laboratory, Universitas Diponegoro*
Jl. Prof. Soedarto S.H., Tembalang Semarang Central Java, 50275 Indonesia
[3]*Graduate School of Engineering and Science, University of The Ryukyus*
Nishihara, Okinawa 903-0123, Japan
[4]*Biology Department, Faculty of Science and Mathematics, Universitas Diponegoro*
[5]*Department of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro*
Jl. Prof. Jacub Rais, Tembalang Semarang Central Java, 50275 Indonesia
Email: maritajoes@gmail.com

Abstract

Anchovy is the main catch and the primary consumption of coastal communities in Indonesia, and its production shows an increase of more than 10% in 2021. Tuban district, in East Java, Indonesia is part of the WPP 712 (Wilayah Pengelolaan Perikanan or Fisheries Management Area) and highly produces anchovies' fisheries. Anchovy has a small size, making it difficult to identify morphologically. This study aimed to genetically identify anchovy samples obtained from North Java (Tuban) waters. Molecular identification was conducted by utilizing Cytochrome Oxidase Subunit I (COI) gene using jg-HCO and jg-LCO primers. This study observed 12 individual samples with 623 base pair sequence length. Five species were obtained, namely four species of anchovies (Encrasicholina heteroloba, Encrasicholina punctifer, Stolephorus waitei, and Stolephorus insularis) and one species of sardines (Dussumieria elopsoides) with 99.84-100% similarity to NCBI sequences data. Anchovies typically have a streamlined body with a slightly compressed shape. Anchovies have cycloid scales, which are smooth-edged and relatively small, ranging from a few centimeters to around 20 centimeters in length. Some of the genus from the Anchovy group are Encrasicholina and Stolephorus. The phylogenetic tree reconstruction leads into four clades with a genetic distance between clades of 17,9-24,5 %. This research provides methods and data on the genetic diversity of anchovies taxa caught in Tuban, East Java. The findings are expected to support promoting new standards for healthier and more sustainable anchovy stocks in the country. Overall, this study contributes to providing valuable insights for fisheries management and conservation efforts in Indonesia.

Keywords: *Anchovies species, molecular, DNA Barcoding, Tuban*

Introduction

Anchovies are an important fishery commodity due to their high demand for aquaculture feed, industrial oil, health supplements, and human consumption (Barange *et al.,* 2014). In addition, these fish are economically significant protein sources for the population. According to Robinson, (2022), anchovies are important small pelagic resources in fulfilling nutrition and protein for people in an area. They are small oily fish which are rich in omega-3 fatty acids and other nutrients (Mattimu *et al.,* 2016), making them a popular food item. Regarding the fishing industry, anchovy fisheries can be found worldwide, with significant fisheries in the Mediterranean, the Pacific, and South America (Checkley *et al.,* 2017). Anchovies are scattered in Indonesian waters, and it is one of the fishery export commodities with a value between USD 5.77-6.92 per kg (Susanto *et al.,* 2017). East Java Province occupies the second position in Indonesia as a region that utilizes it by processing anchovies from its waters (East Java Maritime and Fishery Service Office 2015). Tuban is one of the regencies in East Java that uses anchovies as its flagship product.

Management of sustainable fisheries needs to be carried out to ensure the sustainability of resources. This issue is related to fish as a promising commodity; overfishing has an impact on decreasing yields, competition between industrial fishermen and traditional fishermen, and the management of fishing that is less efficient (Sari *et al.,* 2022). Anchovies as a result of overfishing, which impacts decreasing catches (Sari *et al.,* 2022), as in this study, anchovies

*) Corresponding author

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were caught at juvenile sizes. Anchovy conservation in Indonesia is critical due to the high demand as a food source and their essential role in the marine ecosystem.

Anchovies belong to the class Actinopterygii and are very diverse. Morphologically, anchovy has a small body and a flat shape. The elongated body is equipped with a silver line extending from the head's base to the tail's base. The caudal fin is forked and not joined with the anal fin (Whitehead *et al.,* 1986; Nelson *et al.,* 2016). Anchovies live in small or large groups ranging from a few hundred individuals to millions-living anchovies in coastal and estuary waters (Checkley *et al.,* 2017). Anchovy is part of an assemblage of fish that eat plankton and forage for predatory fish, squid, mammals, and birds. They are important in transferring energy from lower to higher trophic-level organisms (Palomera *et al.,* 2007). Climate change can affect the anchovy populations, such as ocean warming, acidification, nutrient supply, habitat compression, phenology, and demand in the food web (Checkley Jr *et al.,* 2017).

Their small body size makes it challenging to observe morphological differences in anchovies, making it difficult to identify species morphologically. Therefore, it is necessary to identify anchovies using a molecular approach, using DNA barcoding (Jung *et al.,* 2019). Molecular analysis can distinguish between cryptic species of anchovies by looking at differences in DNA sequence (Trivedi *et al.,* 2016). COI (Cytochrome C Oxidase subunit I) is a mitochondrial gene commonly used as a molecular marker for DNA barcoding of some marine organisms such as fish (Liu *et al.,* 2020; Sudibyo *et al.,* 2020; Dwifajri *et al.,* 2022), mollusk (Juniar *et al.,* 2021; Kholilah *et al.,* 2021; Nasution *et al.,* 2021), crustacea (Fajarallah *et al.,* 2019; Ng *et al.,* 2022; Lastria *et al.,* 2023), Echinodermata (Amin *et al.,* 2020; Sulardiono *et al.,* 2022; Nugroho *et al.,* 2022) and corals (Insafitri *et al.,* 2023). The COI gene's DNA sequence can help identify and distinguish cryptic species that may appear morphologically identical.

This study aimed to determine the species identity of Tuban anchovies in seawater based on DNA barcoding of COI. DNA barcoding using the COI gene can be a valuable tool to supplement morphological identification and provide more accurate species identification. In addition, the COI gene can be used to detect cryptic species and better understand the biodiversity of a particular group of organisms, including anchovies. This information can be used to develop conservation and management strategies for this group.

Materials and Methods

This study analyzed 11 samples of anchovies and one sample of sardines from Tuban Regency, East Java, Indonesia (Table 1.). The samples were collected in 2022 from local fishermen (Figure 1). Each sample was preserved in 96% ethanol and stored in a -20 °C freezer until utilized.

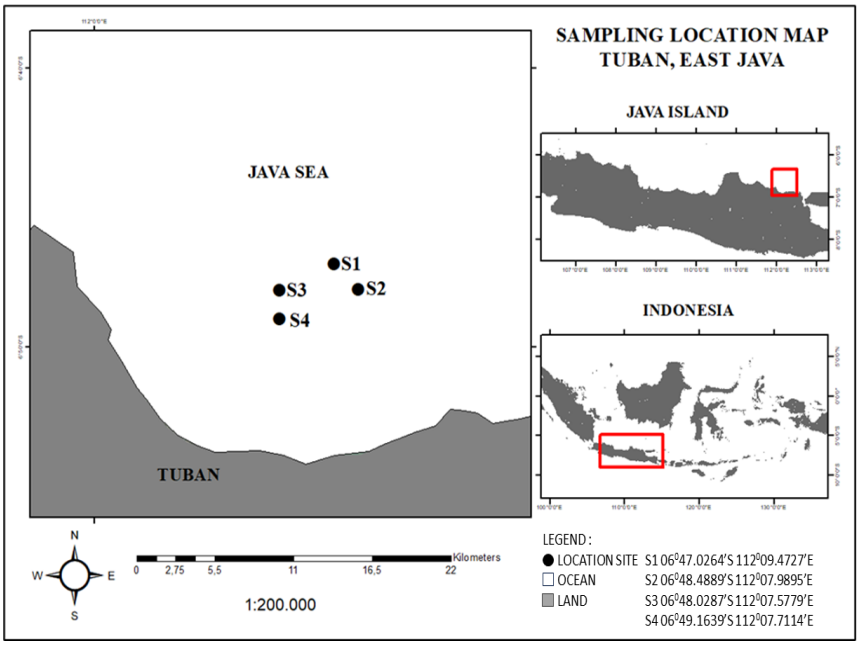

Figure 1. Map of sampling location of 12 anchovy samples in Tuban Regency, East Java Province, Indonesia.

Table 1. The list of anchovy samples used in this study. The table also shows the similarity percentage with NCBI (The National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov) sequences and its Accession Number, including the species name based on the NCBI's sequences.

Sample ID	Similarity	Accession Number	Species
DBP011901	100%	OR487207	*Encrasicholina heteroloba*
DBP011902	100%	OR487208	*Encrasicholina punctifer*
DBP011903	100%	OR487209	*Stolephorus baweanensis*
	100%		*Stolephorus waitei*
DBP011904	100%	OR487210	*Encrasicholina pseudoheteroloba*
DBP011905	100%	OR487211	*Encrasicholina pseudoheteroloba*
DBP011906	100%	OR487212	*Encrasicholina pseudoheteroloba*
DBP011907	100%	OR487213	*Stolephorus baweanensis*
	100%		*Stolephorus waitei*
DBP011908	99,84%	OR492312	*Dussumieria elopsoides*
DBP011909	100%	OR487214	*Encrasicholina pseudoheteroloba*
DBP011910	100%	OR487215	*Encrasicholina pseudoheteroloba*
DBP011911	100%	OR487216	*Encrasicholina pseudoheteroloba*
DBP011912	100%	OR487217	*Encrasicholina pseudoheteroloba*

Extraction was carried out using a 10% of chelex (Walsh *et al.,* 1991). Approximately 2 mm of the sample tissue is inserted into the tube containing 10% chelex, then heated at 95°C for 45 min (Akbar and Aris, 2018). The Chelex was then vortexed and centrifuged to separate the pellet and the supernatant (the clear solution of DNA in the water solution above the pellet)

A portion of the Cytochrome Oxidase Subunit I (COI) gene was amplified via PCR using the primers jgLCO1490: 5'-TITCIACIAAYCAYAARGAYATTGG-3' and reverse primer jgHCO2198: 5'-TAIACYTCIGGRTGICCRAARAAYCA-3' (Geller *et al.,* 2013). The PCR reaction was carried out in 25 µL volumes, using 1.25 µL of DNA template. Each reaction included 12.5 µL MyTaq™ Red Mix (Bioline), 1 µL of each primer, and 9.25 µL ddH2O. The thermocycling profile included an initial denaturation of 95°C for 4 min, 40 cycles of 95°C for 30s, 50°C for 30s, and 72°C for 1 min, with a final extension of 72° C for 10 min. The PCR reactions were checked on 1% agarose gels stained with Florosafe.

Gel electrophoresis was used to visualize the results of the amplification of the COI gene fragment by PCR. This process was carried out using 1% (w/v) agarose gel (Dailami *et al.,* 2021), visualized with UV-transilluminator, and documented with a digital camera. Amplicon was then sent to the Genetika Science Indonesia laboratory for single-pass DNA sequencing employing an ABI 3730xl DNA Analyzer sequence

DNA sequences were cleaned, edited, and aligned using ClustalW in the MEGA X program (Kumar *et al.,* 2018). The clean sequences were then compared to the open database NCBI (*The National Center for Biotechnology Information*; https://www.ncbi.nlm.nih.gov) using the BLAST program (*Basic Local Alignment Search Tool*; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The most similar sequences (*Encrasicholina heteroloba, Encrasicholina pseudoheteroloba, Encrasicholina punctifer, Dussumieria elopsoides, Stolephorus waitei and Stolephorus baweanensis*) from NCBI were downloaded to be aligned with the sample sequences (Table 1.). Three methods were used to generate phylogenetic reconstructions: neighbor-joining and maximum likelihood using MEGA XI (Kumar *et al.,* 2018) and bayesian using BEAST V2.6.7 (Glickman and Dyk, 2007). The neighbor-joining analysis, based on genetic distance, uses the Kimura 2-Parameter model with 1000 bootstrap replicates to assess clade support. The maximum likelihood also used 1000 bootstrap replicates and ranked the Kimura 2-Parameter model with 1000 bootstrap replicates as the best fit to the data. The Bayesian analysis based on posterior probability value of 10.000.000 replicates with Hasegawa-Kishino-Yano (HKY) model.

The out-group used for this phylogenetic reconstruction is *Thunnus albacares*.

Result and Discussion

Species identification

The morphology of several samples obtained from this study is difficult to identify based on morphological identification (Figure 2.). A total of 12 samples originating from Tuban were successfully sequenced. The sequence length obtained using the primers Jg-LCO and Jg-HCO is 623 bp. The sample is identifiedinto six species, *Encrasicholina heteroloba, Encrasicholina pseudoheteroloba, Encrasicholina punctifer, Dussumieria elopsoides, Stolephorus waitei,* and *Stolephorus baweanensis* based on the NCBI database. The similarity percentage ranges from 99,84-100% (Table 1.). The six species obtained were classified into three genera: two anchovies, namely *Encrasicholina* and *Stolephorus*, and one genus of sardines, namely *Dussumieria*. The species of DBP011903 and DBP011907 cannot be ascertained. This could happen because the sequences obtained were relatively short and could not be utilized as an identification key for these samples.

Anchovies typically have a streamlined body with a slightly compressed shape. Anchovies have cycloid scales, which are smooth-edged and relatively small. Encrasicholina species are generally small-sized fishes, ranging from a few centimeters to around 20 cm in length. They have two dorsal fins, one on each side of the body, one toward the front, and one toward the back. Usually found close to the tail, the anal fin, rounded abdomen, a sharp tip of the upper jaw, and forked caudal fin. Encrasicholina species can have a variety of colors, although they frequently have silvery or iridescent bodies with blue or green hues on the upper side. Stolephorus species are generally small-sized fishes, ranging from a few centimeters to around 15 cm in length. Stolephorus species can have a variety of colors, but they frequently have silvery or iridescent bodies with blue or green hues on the top. Stolephorus is characterized by the pointed tip of the upper jaw, the body being convex and rounded with many dark spots under the eyes and on the tip of the lower jaw. Dussumieria species typically have a slender and elongated body shape, similar to other herrings. The abdomen is more forward and rounded, the pelvic scales are W-shaped, and the pelvic and dorsal fins are closer to the tail than the head. The size of these fishes is often between a few millimeters and 15 cm, making them tiny. Dussumieria species can have a variety of colors, although they frequently have a silver or translucent body with blue or green hues on the top side (Whitehead *et al*., 1988).

Identification of genetic characters is usually carried out in important species such as tuna and groupers (Troudet *et al.,* 2017). Meanwhile, anchovy groups include 16 genera and 172 species (Eschmeyer *et al.,* 2021) and most of the groups have a small size thus it is difficult to identify (Gangan and Pavan-Kumar, 2019). This makes the species identification of the anchovy group crucial. Molecular identification of anchovies using the COI locus helps to solve the possibility of identification errors and ambiguous species (Gangan and Pavan-Kumar, 2019).

Phylogenetic tree

The phylogenetic tree of 12 samples and seven NCBI sequences was reconstructed from locus COI sequences with Jg-HCO and Jg-LCO primers, as seen in Figure 2. A total of four clades were formed with a bootstrap value of 45-100%. Phylogenetic construction based on COI sequences found four distinct clusters of anchovies samples taken from Tuban Island-Indonesia (Figure 3.). *Thunnus albacares* sequence was used as an outgroup in the phylogenetic reconstruction. As a result, there are five clades formed, including the outgroup. The first clade consisted of eight samples with two species, *Encrasicholina heteroloba* (DBP011901) and *Encrasicholina pseudoheteroloba* (DBP011904, DBP011905, DBP011906, DBP011909, DBP011910, DBP011911, and DBP011912). The second clade was identified as *Encrasicholina punctifer* (DBP011902) species. The third clade was identified as *Dussumieria elopsoides* (DBP011908), and the fourth clade was identified as *Stolephorus waitei* and *Stolephorus baweanensis* (DBP011903 dan DBP011907).

The first clade identified two species (*E. heteroloba* and *E. pseudoheteroloba*). Both species show 100% similarities with the samples. This result indicates that both species were identified with the same sequence of DNA. Based on the World Register of Marine Species (WORMS, https://www.marinespecies.org), *E. pseudoheteroloba* (Hardenberg, 1933) is an unaccepted name. The accepted name for this species is *E. heteroloba* (Ruppell, 1837). This is an example of the dynamic in the organism taxonomy and classification systems, including marine fishes. Thus, the morphology and molecular identification in marine fishes are still important and must be explored to catalog the diversity of our marine life.

Another example of the double species' name is in clade 4, where the samples were identified as either *S. waitei* or *S. baweanensis*. *S. waitei* is a valid species name, however, *S. baweanensis* (Hardenberg, 1933) is the unaccepted name based

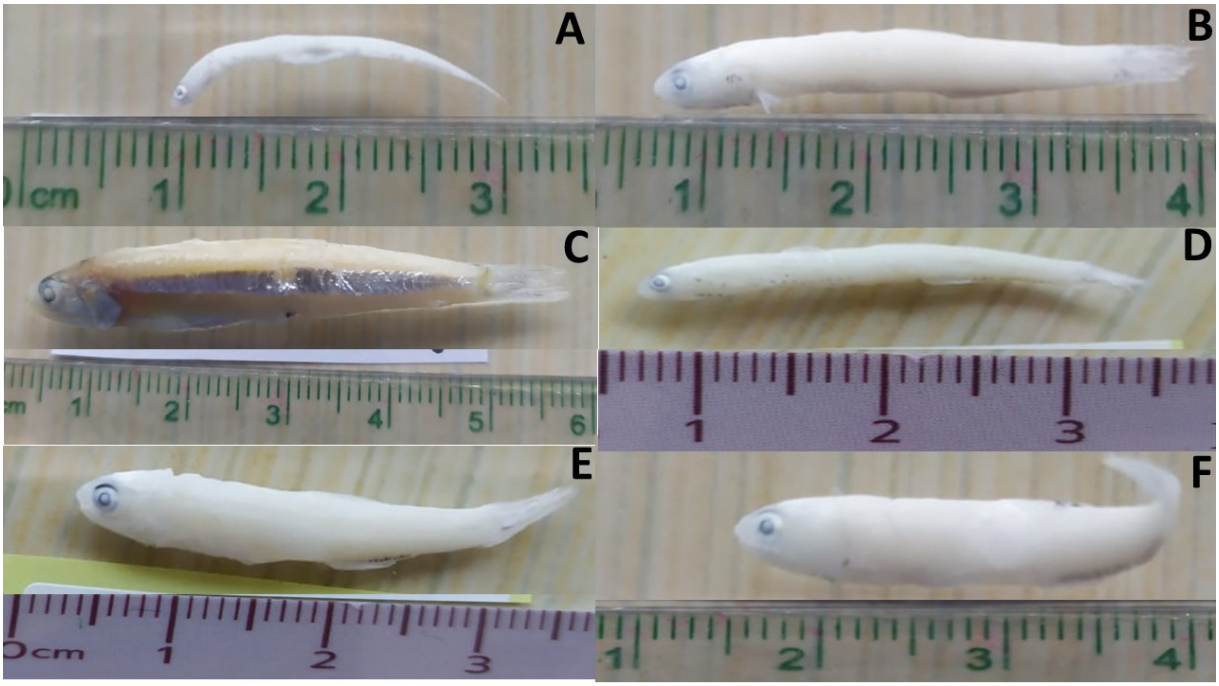

Figure 2. The morphology of several species of anchovies obtained from this study. **A.** *E. heteroloba,* **B.** *E. paseudoheteroloba,* **C.** *E. punctifer,* **D.** *D. elapsoides,* **E.** *S. waitei* and **F.** *S. baweanensis. E. heteroloba* and *E. paseudoheteroloba* later described as single species, *E. heteroloba* and *S. baweanensis* described as *S. insularis* based on the World Register of Marine Species (WORMS, https://www.marinespecies.org).

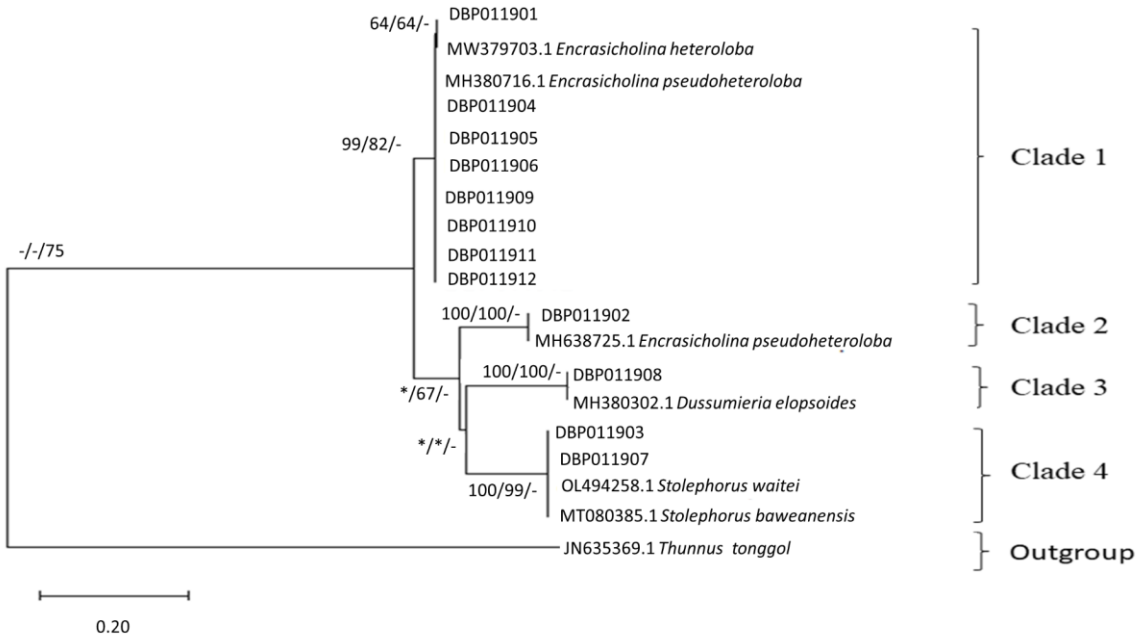

Figure 3. Phylogenetic tree of anchovy species with Neighbor Joining (NJ) topology generated from 623 bp of mtDNA COI gene. Numbers above the major nodes indicate bootstrap support for 1000 bootstrap using neighbor-joining and maximum likelihood, respectively and 10.000.000 replicates using bayesian.

Table 2. Genetic distance between clade of 12 anchovy samples and sardines obtained from this study.

	Clade_1	Clade_2	Clade_3
Clade_1			
Clade_2	0.179		
Clade_3	0.241	0.229	
Clade_4	0.215	0.217	0.245

on WORMS (https://www.marinespecies.org). The accepted name for *S. baweanensis* is *S. insularis* (Hardenberg, 1933). If we aligned both sequences from NCBI for *S. baweanensis* (MT080385.1) and *S.waitei* (OL494258.1), they were identical sequences. The phylogenetic with Cytochrome Oxidase Sub-Unit I (COI) gene obtained from NCBI, shows that some sequences of *S. waitei* and *S. baweanensis* (*S. insularis*) were clade together (accession number OL494258 and MT080385. This shows that molecular identification needs to be supported by morphological identification. The small size and the similarity of the morphological characters between anchovies species can lead to misidentification and, in the bigger context, mislabelling.

Dussumieria elopsoides (DBP011908) known as rainbow slender sardines. *D. elopsidesis* a small, subtropical marine fish from the Family Clupeidae (Whitehead, 1985). Juveniles of *D. elopsidesis* are known to inhabit shallow coastal waters with the estimated total length of these fish being 70-180 mm. They exhibit schooling behavior that helps them avoid predation and improve their foraging efficiency (Fanning *et al.,* 2019; Kaltenberg and Benoit-Bird*.,* 2009). These fish feed mainly on plankton and serve as a food source for predators, thereby sustaining the food chain and ecosystem of the marine environment (Mukhopadhyay *et al.,* 2020; Roy *et al.,* 2018). According to reports, most small pelagic fish stocks, such as sardines, are currently being fished sustainably to underfished levels in the Indo-Pacific marine water (FAO, 2018).

Genetic distance

Genetic distance describes the number of nucleotide bases that differ between individuals. The distance can be seen in Table 2. Genetic distance between clades ranges from 0,179-0,245. A more than 3% genetic distance indicates that each clade is a distinct species (Hebert *et al.,* 2002).

Anchovies are a widespread fish consumption. Therefore, studying the habitat preferences and distribution of anchovies can identify critical areas for their conservation and protection. Several important studies have been carried out to find out what species of anchovies exist in Indonesia, habitat mapping, and maintain sustainability such as taking into account stocks in nature caught at a specific size (such as already matured gonads and spawn) (Yonvitner *et al.,* 2020) and the number of catches must be limited (Yue *et al.,* 2021).

Anchovy fisheries production in Indonesia showing an increase of more than 10% in 2021. Anchovy fisheries in Madura Strait, East Java, are being proposed to get an eco-labeling certificate by the Indonesian government. The East Java fisheries area is part of the WPP 712 (*Wilayah Pengelolaan Perikanan* or Fisheries Management Area). The Indonesian government supports the implementation of the MSC (Marine Stewardship Council) standard to promote healthier and more sustainable anchovy stocks. This research contributes to what species fishermen catch in Tuban, East Java. Genetic studies help to provide genetic data information to support management decisions for anchovy conservation and sustainable use.

Conclusion

The results of DNA analysis showed that the length of the COI gene fragment of the anchovy obtained was 623 base pairs. This study obtained five species from 12 samples, namely four species of anchovies (*E. heteroloba, E. punctifer, S. waitei* and *S. insularis*) and one species of sardines (*Dussumieria elopsoides*) with 99.84-100% similarity to NCBI data. The phylogenetic tree reconstruction leads into four clades with a genetic distance between clades of 17,9-24,5 %. The molecular identification of anchovy groups can help the management authorities for sustainable fisheries in Indonesia.

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