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Molecular phylogeny of anchovy (Clupeiformes: Clupeidae) from southern waters of Lombok using mitochondrial DNA CO1 gene sequences

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Abstract. *Mahrus H, Idrus AA, Zulkifli L. 2022. Molecular phylogeny of anchovy (Clupeiformes: Clupeidae) from southern waters of Lombok using mitochondrial DNA CO1 gene sequences. Biodiversitas 23: 2433-2443*. This study aimed to identify anchovy samples genetically and evaluate their phylogenetic relationship to anchovies using the CO1 genetics markers. Fragment amplification of COI gene anchovy used a pair of COI commercially available primer with a length amplicon of about 693 base pairs (bp), coding for 231 amino acids successfully. The reconstruction of the phylogenetic tree with the neighbor-joining (NJ) method using Kimura 2 Parameters (K2P) by employing a bootstrap of 1000 replications. The genetic distance analysis between 19 fishes species in different families and 13 species in the genera Spartelloides acceded from NCBI indicated the closest distance between anchovy samples from the southern waters of Lombok and *Spratelloides delicatulus* from Australia is 0.02. The comparisons results of homology with the NCBI and the BOLD database show the anchovy sample has similarities to the CO1 sequences of *S. delicatulus* with a similar of 99%. The results of the phylogenetic tree analysis showed that the anchovy samples from the southern waters of Lombok were in the same clade as *S. delicatulus* from Australia. The study concluded a fish sample of anchovy from the southerly waters of Lombok was a closed related species to *S. delicatulus*.

Keywords: CO1, evolution, genetics distance, mtDNA, nucleotide, phylogenetic, *Spratelloides*

INTRODUCTION

The Indonesian islands placed in connecting the Pacific and the Indian Ocean, being Indonesia's sea is a place of exchange for both of them. The Indonesian islands put down interconnect to the Pacific and the Indian Ocean, being Indonesia's sea is a place of interchange for both of them. More than 33,000 fish species in nature are the most speciose group of vertebrates that inhabit nearly all major aquatic habitat types (Helfman et al. 2009). About 70% of them inhabit the Pacific sea waters, which exis as the great sea. The rest is more than 20% of them from the Atlantic Ocean, while 8% comes from the Indian Ocean with the warmest waters characteristics.

Lombok strait, located between Bali and Lombok islands, links the Java Sea to the Indian Seas, well known as one of the dominant main water through flow passages in the Indonesia sea. At the same time, the water exchange occurs in the middle of the Indian Ocean and the Pacific Ocean. Along the south coast of the Sumatra-Java and southern waters of the Lombok area of an increase in water mass (upwelling) in the east season (June to November). Upwelling is the increase inside seawater mass with low-temperature characteristics, high salinity, and rich in nutrients, thereby increasing the amount of chlorophyll in the Indonesian waters that can arise water fertility would cause an abundance of biomass productivity, fertile, and abundant fish (Hendrawan 2011; Eisele et al. 2021; Xu et al. 2021).

The anchovy from the genus Spratelloides (Clupeiformes: Clupeidae) is small pelagic fish, indicators part of the coastal ecosystem, and plays a crucial role in the food webs of continental shelves across the globe because of its abundance (Velasco-Lozano et al. 2020; Eisele et al. 2021). They distribute near the coastal waters, mainly in warm waters, live and swim naturally (Ernawati et al. 2018; Bray 2019). The fish is a species always caught throughout the year using lift nets, which belongs to the purse seine type. The family Clupeidae classification is the most exciting due to the difficulties in diagnosing the Clupeidae and several of their subfamilies (Rogers et al. 2003). Because of the extensive spreading, molecular and morphological diversity of Clupeidae has resulted in an appreciable debate related to taxonomy in recent years which recognizes two suborders currently (Queiroz et al. 2020).

According to this information, taxonomy and identification of small pelagic fish like Clupeidae have always been problematic due to various factors. Most fishes undergo ontogenetic metamorphism, so many characteristics of morphometry change during ontogenetic development (Zhang and Hanner 2011). Moreover, concurrent and different transformations also affect the metamorphosis of morphological features, which causes many species to be controversial in their taxonomy (Templonuevo et al. 2018). The limitation elementary in morphology description systems needs a molecular approach using DNA Barcoding to identify species. DNA

Barcodes CO1 gene is the best solution currently to identify some fishes and other fauna species because it works fast and accurately' results (KeskİN and Atar 2013; Bingpeng et al. 2018; Buckwalter et al. 2019). In addition, the COI gene can identify species which a conservative nucleotide base sequence and only undergo slight variations, deletions, and insertions (Miya et al. 2015; Phillips et al. 2019).

Gene CO1 is one of the protein-coding genes in the mitochondrial genome (Chen et al. 2021). Using Gene CO1 to identify fauna in Indonesia is very limited to date. Research and Development Center for Biology, Indonesian Institute of Sciences reported that fauna in Indonesia already has a DNA barcode as mammals, birds, Komodo dragons, and insect pests (Rahayu et al. 2019). Based on the data reported by The Indonesian Institute of Sciences, it appears that various other species of small pelagic fish in Indonesian waters do not yet have DNA barcodes. The researchers are very interested in researching the genetic analysis of anchovy (Clupeiformes: Clupeidae) of the Coastal Waters of Southern Lombok using the DNA barcode gene CO1. This research purposed to evaluate the phylogenetic relationship of anchovy (Clupeiformes: Clupeidae) from the southern waters of Lombok, using CO1 genetics markers.

MATERIALS AND METHODS

Sampling sites and samples collection

The collection of anchovy samples started from April to June 2021 along the coastal water of south Lombok in three different locations: Tanjung Luar, Selong Belanak, and Sekotong (Figure 1). Overall the research sample amounted to 45 fish samples from three places for the research, and nine fishes were for molecular identification. Fixating all specimens use 96% ethanol, and are deposited in the Immunology Laboratory, Sciences Faculty, University of Mataram. Collecting samples methods use purposively. Identification of all fishes samples morphologically uses methods according to FAO species identification sheets (Whitehead 1985; Lavoue et al. 2013; Hata and Motomura 2017). In addition, this study used 13 *Spratelloides* sp. specimens for examining genetic diversity and phylogenetic relationship analysis. This group of fish is often called anchovy in Indonesia.

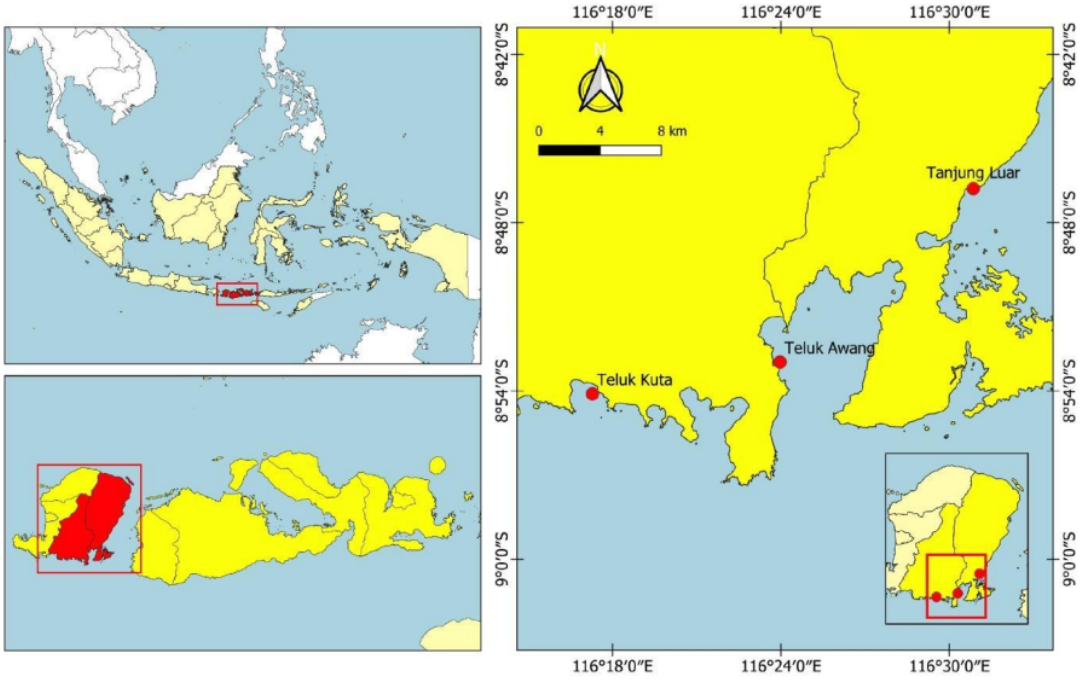

Figure 1. Sampling locations of *Spratelloides* spp. in southern Lombok Coastal Waters. The geographical positions of sampling locations (*) include 1. Tanjung Luar (8°46'47''S, 116°30'51''E); 2. Teluk Awang (8°52'58''S, 116°23'58''E); and 3. Teluk Kuta (8°54'06''S, 116°17'17''E)

DNA extraction, amplification, and sequencing

The amplified CO1 gene use primer pairs: Forward primer FishF1: TCAACCAACCACAAAGACATTGGCAC and Reverse primer FishR1 TAGACTTCTGGGTGG CCAAAGAATCA (Lakra et al. 2011; Bingpeng et al. 2018). DNA is extracted from a small piece of ethanol-preserved muscle tissue, using slightly modified methods according to the standard DNA barcoding methods for fish (Ward et al. 2005; Zhang and Hanner 2011). In this study, approximately 655 base pairs (bp) were amplified from the 5' region of the mitochondrial DNA COI gene. The condition for Polymerase Chain Reaction (PCR) amplification includes 5-minute denaturing step in 35 cycles at 95ºC (30 sec.), 50ºC (30 sec.), 72ºC (50 sec.). The reaction used a total volume of 50 µL containing one µL DNA template, 10 mm Tris-HCl (pH 9), 50 mm KCl, two mm of $MgCl_2$, 0.2 µm of each primer, and 0.2 mm of each dNTP, and the one U Taq polymerase. The PCR products migrated using 1.2% agarose gel visualized under UV light and documented using gel photos (Gel Documentation, UV Cambridge). Good PCR products were sequenced using the Sanger termination dideoxy method conducted by 1st Base, Malaysia through PT Genetika Science Indonesia. The results were sent to researchers in an AB1 file form via email.

Proofreading DNA sequences

The sequencing results in an AB1 file form and then conform between the electropherogram and the DNA sequences obtained. Sequences obtained from a pair of CO1 primers were used as forward and reverse primers, respectively, by performing a reverse complement to use as the reverse. The sequences saved in an AB1 file fare checked for conformity between the electropherogram and the DNA sequences obtained. The sequences obtained from a pair of CO1 primers were used as for and reverse primers, respectively, by performing a reverse complement used as the reverse. The alignment of two sequences used the W cluster menu (Thompson et al. 1994; Larkin et al. 2007). First, nucleotide sequences that do not complement the electropherogram are revised. Next, compare them with the color of each peak in the electropherogram. Finally, the alignment results are combined into one sequence (contig) and saved in a fasta file format. The researchers submitted all successions to dataset GenBank and BOLD system to examine their orthologs and aligned together utilizing the Clustal W (Thompson et al. 1994) in Bioedit software (ver.7.0.4.1; Hall 1999).

DNA sequence homology with NCBI and BOLD data

The homology process compared the sequence with the DNA database at GenBank using BLAST, available on the NCBI website (Boratyn et al. 2013). DNA sequences from the samples entered in the BLAST form available on the web and optimizing the dataset of nucleotide collection by selecting the sequences having high similarity. Homology in the barcode of life database (BOLD) under Ratnasingham and Hebert (2007) used on the https://www.boldsystems.org/index.php/IDS_OpenIdEngine page. The selected database is the COI database up to the species level, and the sequence sample fills in the form provided. Homology results from both the NCBI BLAST and the BOLD System are presented in tabular form and downloaded in fast file format in constructing a phylogenetic tree.

Phylogenetic tree

The phylogenetic tree reconstruction uses the Software of Mega X (Kumar et al. 2018). It used neighbor-joining methods with bootstrapping 1000 replications. This study used the Gene CO1 sequence of 19 populations in different families and 13 species in the genus *Spratelloides* accessed from NCBI for examining genetic diversity and phylogenetic relationship analysis. The species of out-group used as a comparison in the phylogenetic analysis is *Sardinella longiceps* accessed from GenBank. The anchovy species from the southern coastal waters of Lombok has similarities with *S. longiceps*, and it is also abundant in Lombok. The genetic distance calculation uses the same approach to create a phylogenetic tree.

Data analysis

Approximate the sequence divergences among strains conducted DnaSP software ver. 6, based on Juke and Cantor substitution model (Rozas et al. 2003). Estimating the sequence divergences among shear uses DnaSP software ver. 6, based on Juke and Cantor substitution model (Rozas et al. 2017). The estimated first value uses analysis of molecular variance (AMOVA, one package with Arlequin software version 3.0 (Excoffier et al. 2005).

RESULTS AND DISCUSSION

COI gene fragment amplicon

This study succeeded in amplifying the fragments of the COI gene of anchovy from the southern coastal waters of Lombok. It showed the presence of DNA fragments on the electrophoresis gel with a length of up to 693 base pairs (Figure 2). The quality of the PCR product performed is excellent and has sufficient concentration to be used in the DNA sequencing stage. This DNA fragment of 693 bp is almost the same as the research results of anchovy, *S. gracilis* (689 bp), from Cenderawasih Bay of Papua Indonesia (Dailami et al. 2021). Some researchers reported the results of this study were a piece longer than the fragment resulting from the previous studies on Indian marine fishes and Taiwan's fishes species (655 bp), groper *Epinephelus* sp. (526 bp), and anchovy from Indonesia around 650 bp (Lakra et al. 2011; Jefri et al. 2015; Bingpeng et al. 2018; Nuryanto et al. 2019; Tapilatu et al. 2021; Dwifajri et al. 2022). Whereas the other researchers reported the DNA bands size of the anchovy sample obtained was almost the same as those fishes found in the family Labridae (Dailami et al. 2018), marine fishes (Bingpeng et al. 2018), *Rhincodon typus* (Toha et al. 2020), Thunnus (Kolondam 2020), even in the gastropod group (Saleky et al. 2016; Leatemia et al. 2018).

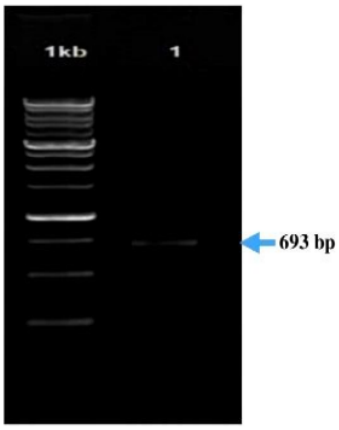

Figure 2. The PCR products of the COI gene from anchovies population of southern coastal waters of Lombok, Indonesia

The difference among the PCR products from different species could occur, and it is a common phenomenon even though these fragments amplify using a similar primer pair. For example, several previous studies reported different lengths of the COI gene from divergent fish species (Lakra et al. 2011; Kusbiyanto et al. 2017; Bingpeng et al. 2018; Nuryanto et al. 2019). It is due to each species having different specific sequences in its genome. The uniqueness occurs and guides to a contrasting size when amplified from other species. DNA sequence difference will affect the results of homology, and the shorter used will have a higher chance of similarity and affect the difficulty to distinguish very similar species (cryptic species).

DNA sequence homology

The coverage results for CO1 gene sequence from 19 species range from 96% to 100% in the NCBI database and BOlD system, which indicates that not all BLAST sequences have the same length as the sample sequence (Table 1). The comparison results of the sequence homology with the NCBI database and the Barcode of Life DataSystems (BOLD) showed that *Spratelloides* sp., a research sample from Lombok, had similar sequences to the COI gene fragment of the *S. delicatulus* species. The similarity level belonging to both species is from 96.78% to 97.68% (NCBI) and 97.63-98.93% in the BOLD system. The assessment for accuracy and reliability of sequences that resulted from the BOLD structure outperformed GenBank for performing comparably of *Spartelloides* sp. (99% and 98%), respectively. From the data, the results for sequence homology with the NCBI and the BOLD system database showed that evidently, a research sample from Lombok had similar sequences to the COI gene fragment of the *S. delicatulus* species with very high similarities (99%). The DNA sequence as big as 693 base pairs codes for 231 amino acids, with the amplified region located at the nucleotide position.

Nucleotide sequences of mtDNA CO1 gene

The DNA sequence obtained after editing and proofreading is 693 base pairs that code for 231 amino acids. This amplified region is located at the nucleotide position 5486 to 6174 of the complete genome of the mitochondrial DNA for *S. delicatulus* (KJ466133.1) with a length of 16.617 base pairs (Lavoué et al. 2007). The analysis of the base composition of the mt DNA COI gene sequence of 19 fish species (Table 2) revealed GC content (51.2%) was higher than AT content (48.8%). The results are almost the same as those found in Australia, Canada, and Cuba fish species (Ward et al. 2005; Hubert et al. 2008; Lara et al. 2010).

Table 1. BLAST analysis of 19 species obtained from the website of the NCBI

Species	GenBank accession number	References	Locations
Anchovy	OM491214	Present study	Indonesia
Spratelloides delicatulus	AP009144.1	(Lavoué et al. 2007)	Australia
Spratelloides gracilis	KP194900.1	(Steinke et al. 2017)	Australia
Spratelloides gracilis	KP194269.1	(Steinke et al.2017)	Australia
Spratelloides delicatulus	KJ466133.1	(Tikochinski et al. 2013)	Australia
Sorsogona tubeculata	KU179075.1	(Rabaoui et al. 2019)	Saudi Arabia
Siganus unimaculatus	AP006031.1	(Yagishita et al. 2009)	Japan
Sorsogona tubeculata	KU499569.1	(Rabaoui et al. 2019)	Saudi Arabia
Apogon hyalosoma	KJ202132.1	(Alcantara et al. 2016)	Philippines
Siganus vulpinus	KM233212.1	(Yan et al. 2016)	Indo-Pacific
Siganus rivulatus	MW376910.1	(S Ali et al. 2020)	Egypt
Heteropriacanthus cruentatus	NC 056807.1	(Badreddine and Bitar 2019)	Lebanon
Thunnus obesus	HM071005.1	(Little et al. 2010)	Indo-Pacific
Sardinella longiceps	MG251950.1	(Sebastian et al. 2020)	India
Thunnus obesus	KY400011.1	(Gong et al. 2017)	China
Thunnus thynnus thynnus	AY302574.2	(Broughton et al. 2006)	USA
Siganus javus	MW008864.1	(Swaminathan et al. 2020)	India
Siganus javus	MW008863.1	(Swaminathan et al. 2020)	India
Upeneus doriae	KU170640.1	(Rabaoui et al. 2015)	Saudi Arabia

The same is also found in fish samples in this study, indicating the amount of GC is more than AT content consisting of GC 51.7% and AT 48.1%. The G and C nucleotides are connected by three hydrogen bonds so that they have a stronger bond than the A and T pairs connecting only by two hydrogen bonds. It will have implications for the denaturation temperature of the DNA sequence. The more G and C, the higher the denaturation temperature.

Based on the bias' use of nitrogen base of positions' three codons, the first codon position of GC content was significantly higher than those other two positions. The position of mt DNA gene in species evolution varies base-mutation selection pressure degrees and use's base bias probable due to mutation pressure in codon's positions. The results would be helpful to determine new genes, molecular genetic's manipulation, and study *S. delicatulus*. However, the codon usage bias of *S. delicatulus* remains unclear in more detail. Li et al. (2021) explain that codon bias usage dominates evolution character in most species. For more than 300-year, the invention of ambiguous taxon implicating evolutionary theory, biogeography, and conservation had an interesting problems debate by researchers (Bickford et al. 2007). From this invention, at least information on dark species DNA sequence can contribute to costly distinctive nature, and even it can provide the foundation of a classification explanation (Jörger and Schrödl 2013).

Comparison of phylogeny 19 species of different families and 13 species of *Spratelloides*

There are 19 species of different families on the phylogenetic tree divided into seven clades (Figure 3) and 13 species in the genera of *Spratelloides* divided into three clades (Figure 4). All species are collectively in a group (clade) except for *Sardinella longiceps* because it is out of the group in the phylogenetic tree (Figures 3 and 4). The clade with the closed genetic distance is between clade 3 with clade 4 (0.08) and includes anchovy from Lombok as the current research sample (Table 3). This analysis shows that clade 3 has a very close relationship with Clade 4 (0.08), and the furthest kinship is between clade 3 with Clade 6 (0.23). In addition, the analysis shows that clade 3 has a very close relationship with Clade 4 (0.08), and the furthest kinship is between clade 3 with Clade 6 (0.23). When viewed from the genetic distance data, it also strongly supports the separation of these clades. The genetic distance between one species and another, both within the same genus and indifferent family, is at least 0.1 except for clade 3 (*Sardinella longiceps*) and clade 6 (*Upeneus doriae*).

In addition, the number of haplotypes, h: 15 with Haplotype diversity, Hd: 0.976 from populations in Figure 3, whereas the samples in Figure 4, h: 11 and Hd: 0.974. It means that both have almost the same haplotype diversity. The results evaluation of departures from neutral expectations using Tajima's D and Fu's showed that first pairwise comparisons were statistically significant among all populations of *S. delicatulus* evaluated in this study. According to Erwin and George (2017), the inhabitants of *Spratelloide*s in this study have low to moderate haplotype diversity may affect their ability to respond to a rapidly changing environment, especially when coupled with increased fishing pressure. This species is not highly migratory, and with increased gene flow through the removal of artificial fences, an efficient fish passage may allow for the buildup of genetic disparity.

Table 2. Composition of nucleotides, A/T, and G/C content in the gene sequence on 19 species obtained from the website of the NCBI

Species	Composition (%)				Total	Content (%)	
	T(U)	C	A	G		A/T	G/C
Anchovy*	26.8	31.5	21.3	20.2	693	48.1	51.7
Spratelloides delicatulus	27.1	31.3	21.2	20.3	689	48.3	51.6
Spratelloides gracilis	26.7	32.1	20.7	20.6	652	47.4	52.7
Spratelloides gracilis	26.7	32.1	20.7	20.6	652	47.4	52.7
Spratelloides delicatulus	26.8	32.9	20.2	20.2	560	47.0	53.1
Sorsogona tubeculata	27.4	29.7	24.7	18.2	691	52.1	47.9
Siganus unimaculatus	27.3	29.4	25.0	18.3	693	52.3	47.7
Sorsogona tubeculata	27.9	29.4	24.5	18.3	678	52.4	47.7
Apogon hyalosoma	26.1	31.9	23.4	18.6	693	49.5	50.5
Siganus vulpinus	27.4	29.3	25.0	18.3	693	52.4	47.6
Siganus rivulatus	29.7	27.8	23.8	18.6	693	53.5	46.4
Heteropriacanthus cruentatus	27.4	30.2	23.4	19.0	689	50.8	49.2
Thunnus obesus	28.5	28.3	24.7	18.5	692	53.2	46.8
Sardinella longiceps	28.1	29.1	23.4	19.3	693	51.5	48.4
Thunnus obesus	28.3	28.5	24.4	18.8	692	52.7	47.3
Thunnus thynnus thynnus	28.6	28.3	24.6	18.5	692	53.2	46.8
Siganus javus	29.3	27.7	24.0	18.9	682	53.3	46.6
Siganus javus	29.3	27.7	24.0	18.9	682	53.3	46.6
Upeneus doriae	29.9	27.7	23.9	18.6	683	53.8	46.3
Average	27.9	29.7	23.3	19.1	678.5	51.2	48.8

Note: *Present study

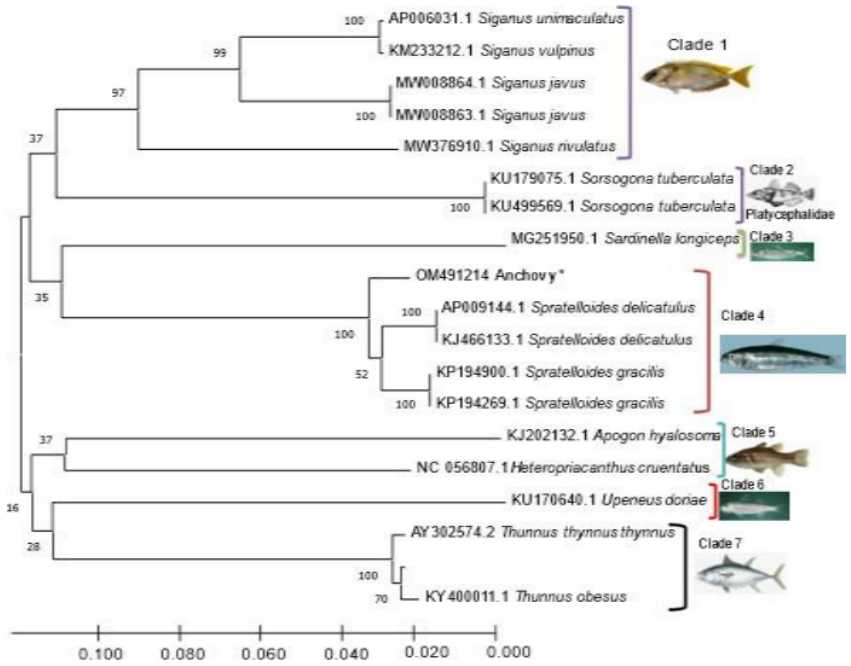

Figure 3. Neighbour Joining tree of CO1 sequences derived from 19 fish species using Kimura 2 Parameters (K2P). *Sardinella longiceps* is a species out of the group. The asterisk (*) denotes the CO1 gene sequence of anchovy obtained from the southern coastal waters of Lombok, Indonesia (Present study)

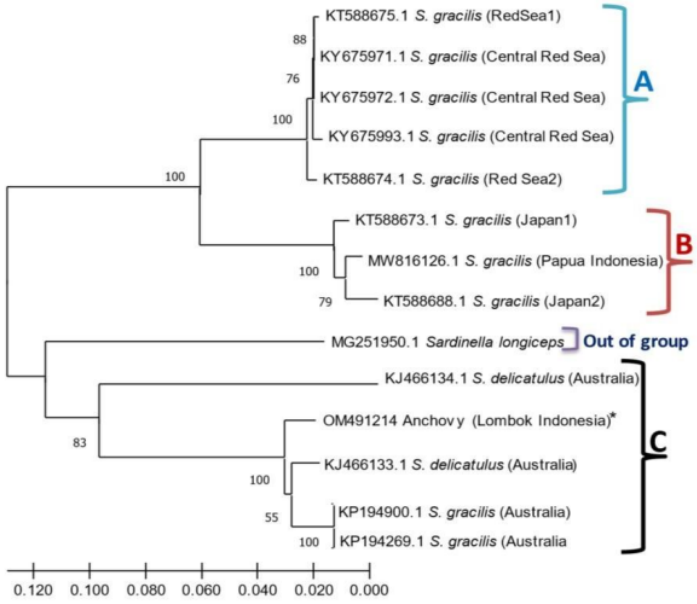

Figure 4. Neighbour Joining tree of CO1 sequences of 13 species in the genera *Spratelloides* and one species out of the group (*Sardinella longiceps*) using Kimura 2 Parameters (K2P). The asterisk (*) denotes the sequence of anchovy obtained from the southern coastal waters of Lombok, Indonesia (Present study)

Table 3. Genetic distance between clades in the phylogenetic tree of 19 individuals

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7
Clade 1							
Clade 2	0.13						
Clade 3	0.12	0.16					
Clade 4	0.13	0.10	**0.08**				
Clade 5	0.14	0.17	0.21	0.12			
Clade 6	0.12	0.16	**0.23**	0.10	0.21		
Clade 7	0.13	0.13	0.10	0.12	0.14	0.10	

Based on a phylogenetic tree of 13 species in the genera *Spratelloides* and 19 species of different families showed the same result, namely fish sample (anchovy) is in the same clade as *S. delicatulus*. In addition, haplotype diversity among 13 species in the *Spartelloides* and 19 species is also high (0.976), and nucleotide diversity (π) = 0.155. According to Nei (1987), the haplotype diversity values ranged from 0.80000-1.0000 in the high category, 0.50000-0.70000 in the medium level, and 0.10000-0.40000 in the low level. It indicates that the successful population size of the *Spratelloides* is high, resulting in high haplotype diversity.

Effectively population size shows the number of individuals who do marry in a population. Therefore, the more individuals that mate, the higher the probability of genetic variation that will form. Ely et al. (2005) stated that even in a local area with a high diversity of haplotypes can emerge. From the values of haplotype diversity and nucleotide diversity obtained, it showed the genetic and nucleotide diversity of anchovy from the southern waters of Lombok is higher than other marine fish species such as longtail tuna on the west coast of India at 0.0998 and 0.0187 (Kunal 2014). Meanwhile, in Indonesia, it is almost the same as an average of 0.995 and 0.018 (Willette et al. 2016; Al Malik et al. 2020). This high diversity of longtail tuna haplotypes is due to the large population size and incidence of interbreeding between individuals.

Figure 4 explained with the understandable result that the fish sample has a closer relationship with the *S. delicatulus* from Australia. The overall mean genetic distance in 13 individuals in the genera of *Spratelloides* population is 0.15±0.01. The genetic distance between groups of *S. delicatulus* samples in Austalia (Pacific Ocean) is 0.082 (group C). The higher the distance value obtained from the two samples group, *S. gracilis* from Japan is 0.016 (group B) and Read sea is 0.004 (groups A). The closer diversity of the tree sample groups is *S. gracilis* from Japan, including from Indonesia (Group B). *S. delicatulus* from sample group C (Southern coastal waters of Lombok and Australia) have a further genetic distance than sample groups B.

Similar results also happened on genetic distance for 13 species in the genera of *Spratelloides* showed anchovy sample from Lombok has the closest kinship to *S. delicatulus* (KJ466133.1) from Australia with a genetic distance of 0.022 (Table 4). Low genetic distance value between sample groups, showing the proximity of the sample groups. It means that all samples in groups are geographically not limited from one to another other. This situation causes the migration process. Next, gene exchange between sample groups happens. The value of the genetic distance of anchovy from Lombok is relatively equal to the genetic distance of *S. delicatulus* from Australia.

Gobin and Warwick (2006) reported the same phenomena in Nematode species diversity. However, it was similar for the northern and southern temperate (the UK and New Zealand) and the tropical area (Trinidad and Tobago). The same results also happened on homology comparisons from the NCBI and the BOLD database, which also shows the species *S. delicatulus* and *S. gracilis* from Australia appeared that allele (haplotype) diversity values 0.900±0.161. According to Nei (1987) and Li and Sadler (1991), this study showed the high genetic diversity, low nucleotide diversity, and the very close relationship of the sample fish with all species in the genera of *Spratelloides*.

Spratelloides delicatulus is one of four species in the genus *Spratelloides*. The complete taxonomy of *S. delicatulus* includes kingdom Animalia, phylum Chordata, class Actinopterygii, order Clupeiformes, Family Clupeidae, Subfamily Dussumieriinae – round herrings, and genus *Spratelloides* (Bennett 1832) (Yahnke et al. 2013; Bray 2019). Queiroz et al. (2020) reported that *Spratelloides* are closely related to *Jenkinsia* and *Chirocentrus dorab*. *Spratelloide gracilis* and *Chirocentrus dorab* belong to Family Chirocentridae, while *Jenkinsia lamprotaenia* and *S. delicatulus* as Family Clupeidae. Preliminary research reported different results the species in the genera *Spratelloides* belong to Family Clupeidae (Lavoue et al. 2013).

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Table 4. Genetic distance for 13 species in the genera of *Spratelloides*

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. OM491214 Anchovy (Indonesia)*												
2. MW816126.1 *S. gracilis* (Indonesia)	0.23											
3. KT588688.1 *S. gracilis* (Japan2)	0.23	0.02										
4. KT588673.1 *S. gracilis* (Japan1)	0.22	0.01	0.02									
5. KT588675.1 *S. gracilis* (RedSea1)	0.22	0.10	0.10	0.10								
6. KT588674.1 *S. gracilis* (RedSea2)	0.22	0.10	0.10	0.10	0.01							
7. KY675971.1 *S. gracilis* (Central RedSea)	0.22	0.10	0.10	0.10	0.00	0.00						
8. KY675972.1 *S. gracilis* (Central RedSea)	0.22	0.10	0.10	0.10	0.00	0.00	0.00					
9. KY675993.1 *S. gracilis* (Central RedSea)	0.22	0.10	0.11	0.10	0.00	0.01	0.00	0.00				
10. KJ466134.1 *S. delicatulus* (Australia)	0.20	**0.27**	0.27	0.27	0.23	0.22	0.23	0.23	0.23			
11. KJ466133.1 *S. delicatulus*(Australia)	**0.02**	0.22	0.22	0.22	0.22	0.22	0.23	0.22	0.22	0.19		
12. KP194900.*I S. gracilis* (Australia)	0.03	0.23	0.24	0.23	0.23	0.23	0.23	0.23	0.22	0.17	0.02	
13. KP194269.1 *S. gracilis* (Australia)	0.03	0.23	0.24	0.23	0.23	0.23	0.23	0.23	0.22	0.17	0.02	0.0

Note: *Present study

This research also informs that the anchovies samples collected have similar morphological characteristics. Initially, this research method relied on the collection of anchovies for other purposes. The study aimed to identify anchovy samples genetically and evaluate their phylogenetic relationship using the CO1 genetics markers. We have collected the fish samples by utilizing a piece of special fishing gear (Stationary lift net) with a small (16 mm) mesh size, under that recommended by Alba et al. (2016). The best net mesh size is 16 mm for sustainable fishing of relatively adult-sized anchovies and white sardine resources. Using this net is intended not to catch species outside of anchovies, but we were unable to avoid it. The fact is, there are other fish species from different families, namely *Sardinella*. In addition, anchovies are not one species but more than one species. Therefore we decided to take the most dominant sample. Identification of anchovies samples based on morphological characteristics has a high level of difficulty and must be careful in identifying. The struggle has also occurred in determining sardine, namely the loss of diagnostic property, e.g., color and physical damage to the fish (Labrador et al. 2021).

Morphological characteristics of anchovies samples are as follows: a cylindrical body shape in the color green with blue reflections due to a silver-colored longitudinal stripe that runs from the base of the caudal fin, the rounded abdomen, the average body length is 15 cm, and covered by the thin scales in texture and easily come off. In addition, it has a pointed snout with a large mouth with the upper jaw extending well behind the eye, the lower jaw is shorter than the upper jaw, and tiny sharp teeth belong to both jaws. In addition, the prominent features are slightly grayish' color anchovy flesh and have a hint of red (Figure 5). These characteristics are similar to the preliminary research reported by several researchers (Ishimori et al. 2015; Alba et al. 2016; Bray, 2019; Navarathne et al. 2019; Lee et al. 2020; Pham et al. 2020).

After identifying the fish samples using the CO1 gene sequence, they were similar to *S. delicatulus* from Australia. However, it is not enough to use the CO1 gene only reported by some researchers. For example, Nakahara and Muraji (2008) used COX I and COX II, Liu et al. (2017) used 16S rDNA. Taxonomic reviews recognized the genus worldwide reveal four species as valid in the genus *Spratelloides* Bleeker, 1851: *S. delicatulus* (Bennett 1831), *S. gracilis* (Temminck and Schlegel 1846), *S. lewis* Wongratana 1983, and *S. robustus* Ogilby, 1897 (Whitehead 1985).

Aside, the sequence of the mtDNA COI gene can estimate genetic separation between the *Spratelloides* species from various locations. Morphological characteristics of the anchovy sample have a very high dive. The difficulty has also occurred in determining sardine, namely the loss of diagnostic property, e.g., color and physical damage to the fish (Labrador et al. 2021). These characteristics are similar to the preliminary research reported by several experts (Bray 2019; Lee et al. 2020; Pham et al. 2020). Therefore, it is not enough to use the CO1 gene only reported by some researchers. For example, Nakahara and Muraji (2008) used COX I and COX II, and Liu et al. (2017) used 16S rDNA.

Figure 5. Anchovy samples in the southern coastal waters of Lombok, Indonesia

The applicability test of a molecular clock for estimating the age of the genus *Spratelloides* by posting the pieces of information onto the independently obtained phylogeny concludes the evolution of species-specific variation for habitat type and fighter caste phragmosis. Maximum-likelihood analysis of both the nucleotide and protein sequences from multigene data holding up single geography is the best approximation of the true phylogeny among the alternatives examined. The fragment of the COI gene is very effective in identifying marine fish species, especially *S. delicatulus*. Several previous studies have also succeeded to identify fish species from the genus Mystus (Pramono et al. 2017), family Labridae (Dailami et al. 2018), whale shark Rhincodon typus (Toha et al. 2020), tilapia fish (Dailami et al. 2021), grouper fish (Tapilatu et al. 2021; Dwifajri et al. 2022), and anchovy family Engraulidae (Afrand et al. 2020; Dailami et al. 2021). The progress of using molecular methods nowadays can identify fish species starting from eggs and provide valuable information for protecting the spawning ground of economically treasures fish and managing fishery resources (Hou et al. 2022).

According to analysis for all parameters of this study, anchovy from the southern coastal waters of Lombok has a kinship relationship closely with *S. delicatulus* (KJ466133.1) from Australia. The factor suspected of having a strong influence is the movement of seawater masses or more popularly called ocean currents accordance with several previous research. For example, Arlindo's transport is a factor that plays a significant role in determining the presence and distribution of marine organisms (Safitri et al. 2010). Arlindo is the sea current coming from both Oceans, the Pacific and the Indian Ocean, entering to Indonesian Ocean through the straits and going out via the Indian Ocean, causing the sea-level difference between the two oceans. The current coming from both oceans will change when it passes through Indonesian waters and then moves to the Indian Ocean (Anggraini et al. 2019). The seawater mass in the South Lombok waters comes from the Australian waters in the Pacific Ocean entering through the Timor strait, impacting the same marine organisms available in both locations (Lombok and Australia). Kang et al. (2012) also reported almost the same thing as the octopus populations around the Korean Peninsula and China evolved separately with the limited genetic exchange by ocean currents. The other study also reported that spawning migration activity for a long time affected by the Indonesian flow of ocean currents caused the phylogeny tree to have the proximity of some of these seas species (Jefri et al. 2015).

In conclusion, the results of DNA mitochondrial CO1 gene analysis find the length of the COI gene fragment from anchovy sample from southern waters of Lombok obtained was 693 base pairs encoding 231 amino acids. According to the results of homology with the NCBI and BOLD databases, this sample is *S. delicatulus* with 98-99% similarity. Furthermore, the phylogenetic relationship also supports the homology results indicating that this sample is in the same clade as the *S. delicatulus* sequence (KJ466133.1) from Australia and has a kinship relationship very closed. Therefore, using the CO1 gene sequence is very effective in identifying the anchovy (*S. delicatulus*) because it is easy, quick, and accurate. It is the first study reporting on the genetic diversity and phylogenetic reconstruction of anchovy from the southern coastal waters of Lombok constructed on a CO1 genes partial sequence. Even though the genetic diversity of anchovy on Lombok island is relatively low, decreasing the diverseness due to habitat demolition and over-exploited has been unavoidable. The study showed a molecular technique-CO1 gene partial sequence, which helps the identification results build on a morphological approach in anchovy found in Lombok island.

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Molecular phylogeny of anchovy (Clupeiformes: Clupeidae) from southern waters of Lombok using mitochondrial DNA CO1 gene sequences

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11