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DNA BARCODING IN THE VALIDATION OF SCAD SPECIES IDENTIFICATION (GENUS: DECAPTERUS) IN AMBON

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Abstract: The limitations of morphology-based identification systems and the decreasing number of taxonomists necessitate a molecular approach for species recognition and identification, with DNA barcoding emerging as an efficient solution to some of taxonomy's challenges. This research aimed to identify several scad species found in the waters surrounding Ambon both morphologically and molecularly through DNA barcoding. Fish samples were collected from January to June 2018 at the Mardika fish market in Ambon, initially analyzed morphologically, and subsequently validated using the DNA barcode method. Both analyses were conducted in the Molecular Biology Laboratory at the Maritime and Marine Science Centre of Excellence of Pattimura University. Morphological identification revealed six species of scad: *Decapterus macarellus, D. macarellus* (suspected), *D. macrosoma, D. macrosoma* (suspected), *D. russelli* (suspected), and *D. kurroides,* with four samples per species collected, resulting in a total of 24 samples analyzed. DNA barcoding identified only four species: *D. macarellus, D. macrosoma, D. russelli,* and *D. kurroides*, with identification percentages ranging from 99-100%. After validation, the confirmed scad species found in Ambon's waters are D. *macarellus, D. macrosoma, D. russelli,* and *D. kurroides,* highlighting that DNA barcoding serves as a complementary method that can reinforce morphology-based identification quickly and accurately.

Keywords: *Decapterus, identification, morphological, molecular.*

1. Introduction

Ambon is a small island in Maluku Province adjacent to Banda Sea. Ambon waters are rich in marine biodiversity, where both non-fin-fish and finfish are widely found, including the small and large demersal and pelagic Fish (Rijoly 2016; Limmon et al., 2017a, 2017b). However, there is a pressing need for updated information to effectively manage these resources. The lack of primary data about the diversity of Fish in Ambon inhibits the marine resource management in the area.

Small pelagic fish such as scad (genus: Decapterus) are predominant species in the area. Despite the substantial catches of scad by fishermen in these waters, there has been insufficient identification of the specific varieties present due to inadequate primary data about the variations of Scad in Ambon's waters or even Maluku's waters.

The limited morphology-based identification system and the lack of taxonomists require the use of molecular approach for the introduction and identification of an organism (Steinke et al., 2009 cited in Zhang & Hanner, 2011). Molecular identification uses DNA pattern that has been proven accurate, relatively easy, and fast compared to conventional methods (Ciardo et al., 2006). DNA barcoding using a short and standardized gene area proposed by Hebert et al. (2003) has been proven helpful in identifying ambiguous taxonomies. The DNA Mitochondria (mtDNA) is a string of DNA passed on by a female parent and is appropriate for analyzing the offspring of a species with a high rate of similarities (Wallace 1997; Syafrina 2011). One of the mtDNA segments usually used as a species marker is cytochrome oxidase I (COI), a mitochondria genome popularized by Hebert et al. (2003). mtDNA genomes are used for biogeography analysis and their systematics frequently diverges from morphology. According to Syafrina (2011) morphological characters usually show similar types of a phenomenon but are genotypically different (cryptic species). The identification of cryptic species has to undergo various taxonomy protocols based on the morphological characters which requires detailed process and longer time (Costa & Carvalho 2007).

Zhang & Hanner (2011) stated that due to high-efficiency rate in species identification, a few ichthyologists recommended using DNA barcode in the formal description of a species (Victor, 2007; Astarlon et al., 2008), since it reinforces quicker and more accurate morphological identification (Lahaye et al., 2008). Lack of information and data regarding scads variety in Ambon combine morphological analysis and DNA barcoding approach in this research. To date, the results of research conducted in Ambon and Maluku by using DNA barcode method have been applied to marine biotas such as marine fish larvae in the Banda

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Sea (Wibowo et al., 2018), coral reefs fish (Limmon, et al., 2017; Limmon et al., 2019), large pelagic fish (Akbar et al., 2018), and hetero-branch (Nimbs et al., 2020). In this research, species variation of scads in Ambon waters was identified by morphological analysis and validated by DNA barcoding approach.

2. Method

Sampling

Scad samples were obtained from Mardika fish market from January to June 2018. Five specimens were collected for every scad species. The samples were photographed, and the scads' body tissue extracted and inserted into a 1.5 ml microtube screw cap filled with 95% alcohol to be stored at -20℃ as voucher specimens.

Morphological Analysis

Morphological identification of all the fish specimens was conducted based on Trautman's method (1957) as cited by Lagler et al. (1977) and Rijoly (1987), including the use of morphometric or meristic calculations. The morphological characters were then compared with the identification key as determined in the reference books (Norman 1935; Genisa 1998; Carpenter & Niem 1999; Cayetano & Honebrink 2000; Golani 2006; Sakinan & Orek 2011; Abdussamad et al., 2013; Dahlan et al., 2014).

DNA Extraction, Amplification, and Sequencing

DNA extraction was done following the Spin-Column Protocol from the Qiagen DNeasy blood and tissue kit as this method offered simpler and more efficient DNA extraction. DNA fragments of the COI gene were amplified in 50µl of PCR reaction which consisted of25µl of Toptaq Master Mix, 10 µl DNA template, and 1µl for every primer [10pmol/µl] and 11µl of Nuclease-free water (H2O). The primer used for the amplification of the DNA fragment of the COI gene was FishF2_t1 (5'TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3') - FishR2_t1 (5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3') and VF2_t1 (5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3') - FR1d_t1 (5' CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA 3'). The PCR amplification process was performed based on Steinke et al.'s (2016) theory, starting with initial denaturation (Hot Start) at 94˚C (10 minutes), and the 40 cycles, which comprised of denaturation at 94˚C (40 seconds), annealing of the primer at 51˚C (40 seconds), elongation at 72ºC (60 seconds), and final elongation for 5 minutes. The PCR was visualized on 2% agarose gel with electrophoresis. The DNA sequencing was done at Macrogen in Korea.

DNA Sequence Analysis

The DNA sequence data obtained from the Macrogen Company in Korea were analyzed using the BLAST program at http://BLAST.ncbi.nlm.nih.gov to identify the similarities/alignment between the nucleotide sequence from this research (Query). Meanwhile, the nucleotide sequences in the gene bank (Subject) were determined using Nucleotide BLAST. After the analysis, species were determined based on the "identified" percentage, with species identification relying on sequence similarity (Song et al., 2008). A higher "identified" value indicated more accurate species identification. The genetic difference value is >2% for intraspecific variation or <2% for interspecific variation, accounting for the possibility of hybridization (Victor et al., 2015).

Genetic and Phylogenetic Distance Analysis

Genetic and phylogenetic distances were analyzed using Molecular Evolutionary Genetics Analysis - MEGA X software (Kumar et al. 2018). Analysis of genetic distance between similar specimens (intraspecific), between specimens of different species and also phylogenetic were performed using based on the Kimura 2 parameter (K2P) model (Kimura, 1980). The results of the phylogenetic analysis are described through a phylogenetic tree created using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). The branching (nodes) on the phylogenetic tree was assessed based on the appearance of bootstrapping analysis with 1000 replications/repetitions (Felsenstein, 1985 in Ran et al. 2020).

3. RESULTS AND DISCUSSION

The Number of Species identified Using Morphological Approach

In the morphological analysis, six species were identified from 24 samples of scad found in Ambon waters. However, three of the six species were suspected as other species (* sign) due to several different characteristics compared to referral material. These six species were then labeled as: *D. macarellus* (ID number RP1-RP4), *D.macarellus** (ID number RP5-RP8), *D. macrosoma* (ID number RP9-RP12), *D. macrosoma** (ID number RP13-RP16), *D. russelli** (ID number RP17-RP20) and *D. kurroides* (ID number RP21-RP24). The morphological characteristics of *D. macarellus, D. macrosoma* and *D. kurroides* are similar to those species in the referral material. Wherea, the suspected species *(D. macarellus*, D. macrosoma*, D. russelli*)* do not resemble the reference as they have slightly different characteristics.

The difference in morphological characteristics found on *D. macarellus* (suspected) lies in the edge of the posterior upper jaw which is rather sunken compared to the references that seem flat (Figure 1). The differences in morphological characteristics that can be found in *D. macrosoma* (suspected) can be seen in the darker upper body with larger body size, pupil, and eye diameter than *D. macrosoma* (Figure 2).

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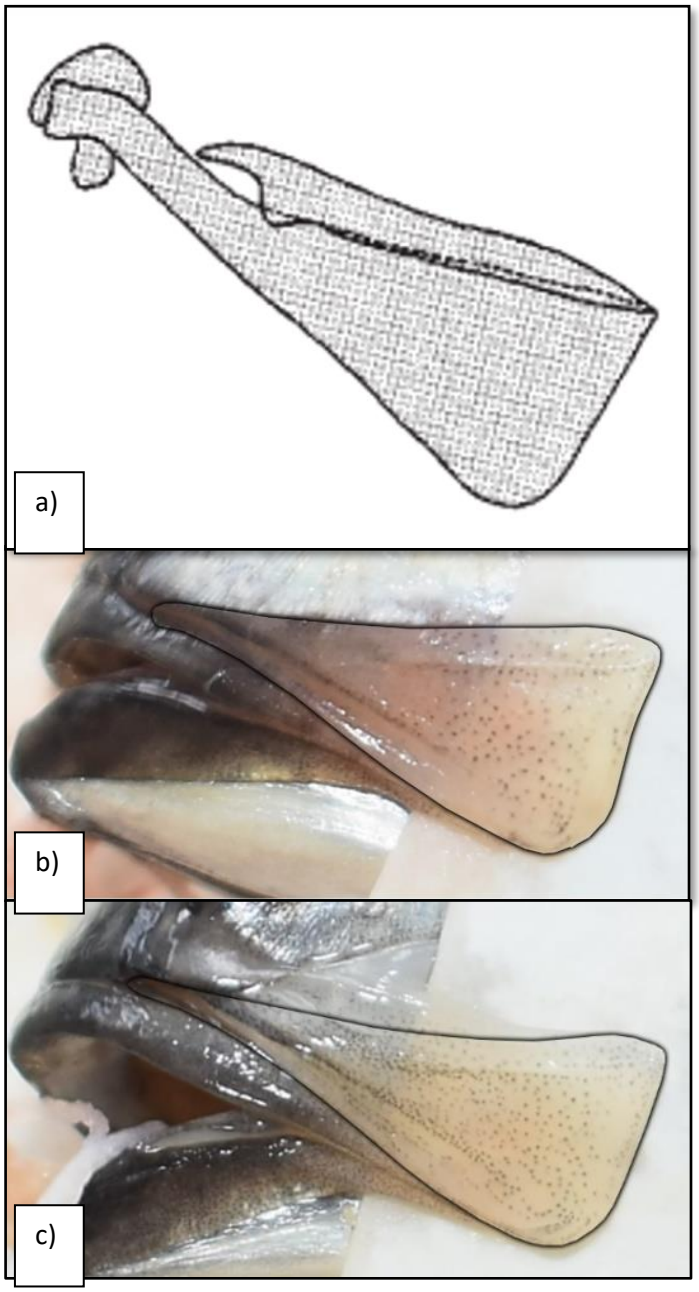

Figure 1. Edge of the posterior upper jaw of *D. macarellus,* a). Carpenter & Volker (1999), *b).* Current research (Suspected) c). Confirmed *D. macarellus.*

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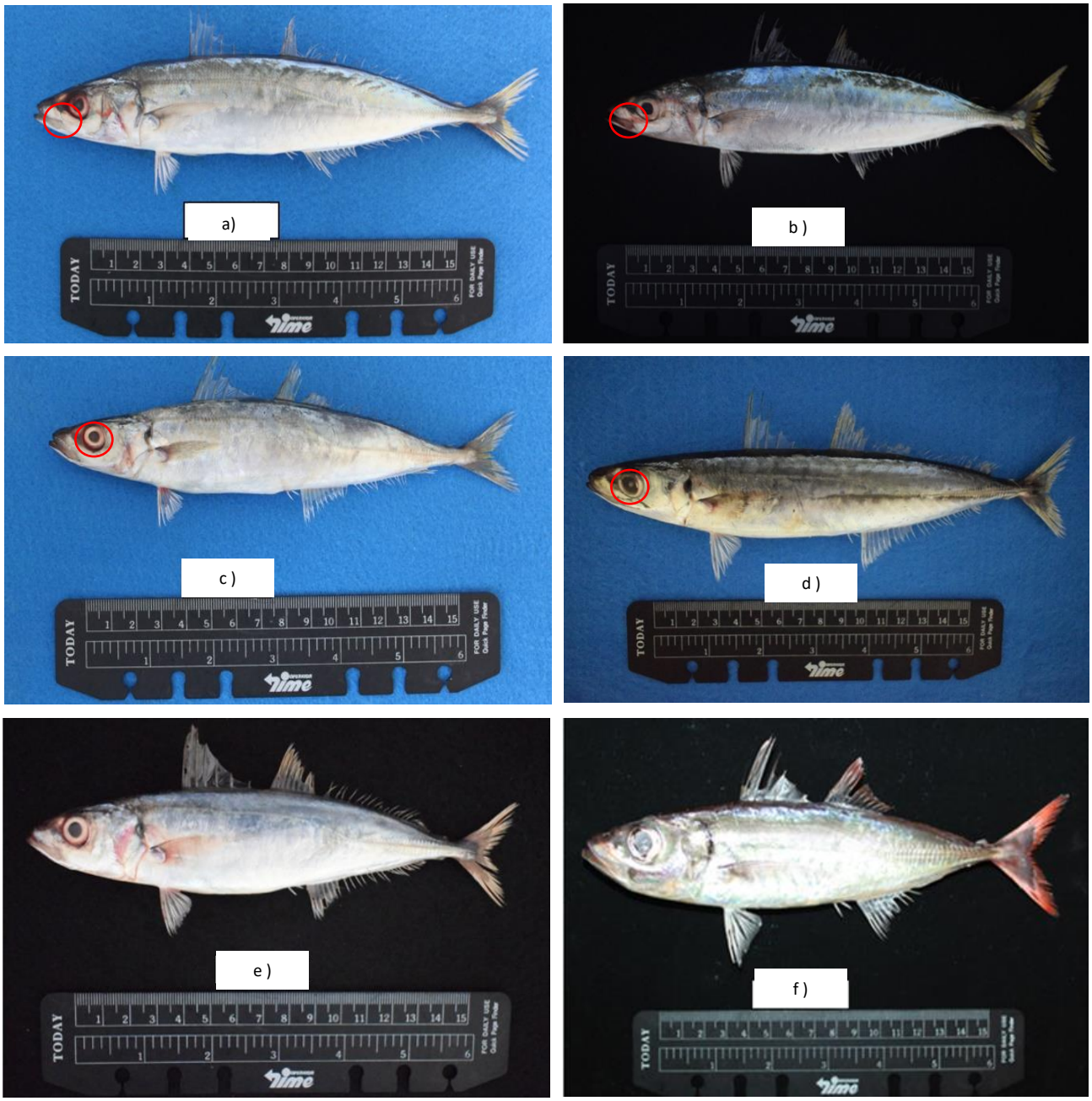

Figure 2. Body differences; a) *D. macarellus,* b) *D. macarellus*,* c) *D. macrosoma*, d) *D. macrosoma**, e) *D. ruselli*,* and f) *D. kurroides*

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The morphological differences of *D. russelli* (suspected) are in the number of filters on the lower gill that range from 26-28. Meanwhile, the reference has between 30-39 (Table 1). The morphological characters of all six species of scad (genus: *Decapterus*) are presented in Table 1.

Table 1. Results of the morphological identification of Scads (genus: Decapterus*)*

morphological characters	*D. macarellus*	*D. macarellus (suspected)*	*D. macrosoma*	*D. macrosoma (suspected)*	*D. russelli (suspected)*	*D. kurroides*
Dorsal Fin (D)	VIII, I 32-36	VIII, I 32-34	VIII, I 34-36	VIII, I 34-37	VIII, I 29-32	VIII, I 29-31
Anal Fin (A)	II, I 28-30	II, I 28-29	II, I 28-30	II, I 28-30	II, I 24-26	II, I 23-24
Pectoral Fin (P)	19 - 20	20	22	20	20	20
Pelvic Fin (V)	I 5	I 5	I 5	I 5	I 5	I 5
Fin Length/Head Length	59.90-65.06%	60.64-63.24%	60.34-72.45%	63.16-67.51%	90.05-95.61%	86.36-96.62%
Scales (LL)	110 - 113	107-132	113-120	115 - 120	96 – 98	83 - 85
Scale (Curved)	50 - 62	55 – 72	60 – 62	58 - 64	56 -58	50-52
Scute (Curved)	0	0	0	0	0 – 2	0
Scale (Straight)	18 - 30	18-22	18 – 22	14 - 24	0 – 2	0
Scute (Straight)	26 -37	32 - 40	34 – 38	34 - 40	38 – 40	33 - 34
Gill Filter (Upper)	11 - 13	12 - 13	10 – 12	11 - 12	10 – 12	11 - 12
Gill FIlter (Lower)	34 - 40	38 -39	34 – 35	33 - 34	26 -28	27 - 30

The presence of "suspected" status for some species of scad appears as this species could not be precisely determined due to some different morphological characters. In general, fish with broader r diversity in their populations than other vertebrates and are more vulnerable to morphological variations influenced by the environment (Wimberger, 1992, cited in Sen *et al*., 2011). Morphological structures, such as plasticity, shape, size, and colour pattern on the body can change from adaptation (Ward et al., 2008; Lakra et al., 2009). Species identification is usually limited based on the status of the distinctive morphological characters (Wiens & Servedio 2000).

Identifying all species shows overlapping characteristics between *D. macrosoma* and *D. Macarellus* which made the identification blurry. Carpenter and Volder (1999) stated that *D. macarellus* and *D. macrosoma* are difficult to identify. Meanwhile, *D. ruselli** and *D. kurroides* species have distinctive morphological characteristics.

The morphological differences are the determinant in the identification success. The "suspected" scad are other species that are not included in the identification book (Carpenter & Niem 1999). Therefore, further research need to be conducted to precisely identify the "suspected" (ambiguous) scad. The constraint in morphological identification system and the lack of taxonomists require a more reliable approach for species identification (Steinke et al., 2009) and precise results (Wong, 2011).

The Number of Species Identified Using Molecular Approach

DNA fragments from COI gene for 24 specimens of scads were successfully amplified using primer FishF2_t1 - FishR2_t1 and VF2_t1 - FR1d_t1 with the length of DNA fragments ranging from 700 to 800 bp. The BLAST analysis on the sequence of nucleotide base COI gene DNA fragments from 24 scad specimens resulted in four species of scads (genus Decapterus) whose identification percentage number ranged from 99 to 100%. The four species are *D. macarellus* with an identification percentage of 99.83-100% (mean of 99.98%), *D. macrosoma* with an identification percentage of 99.82-100 (mean of 99.97%), *D. russelli* with an identification percentage of 99.64-99.83% (mean of 99.79%), and *D. kurroides* with an identification percentage of 99.23-99.84% (mean of 99.57%).

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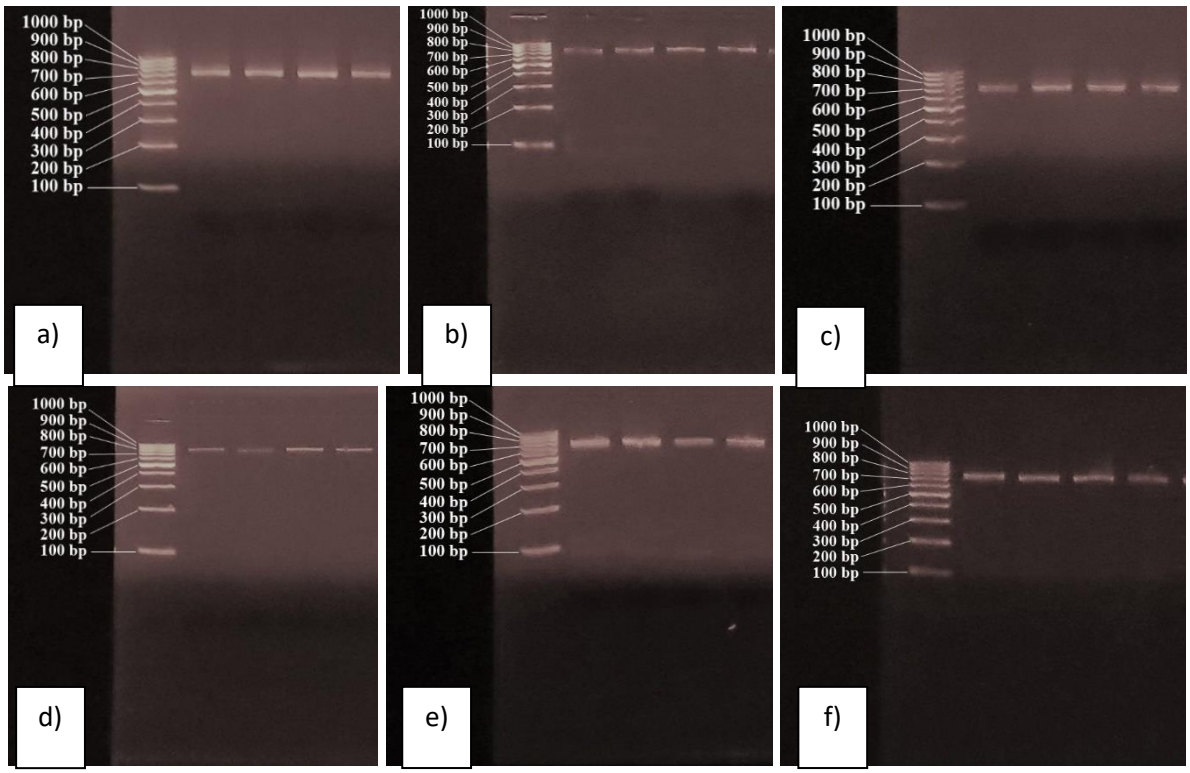

Figure 3. Gel image of PCR product; a) *D. macarellus,* b) *D. macarellus*,* c) *D. macrosoma,* d) *D. macrosoma**, e) *D. ruselli*,* and f) *D. kurroides*

Table 2. Results of DNA sequencing on the DNA fragment of the COI gene on all six species of scads (genus: *Decapterus)*

Sequence ID	Species	Query Cover (%)	Identified (%)	Accession Number
RP1	*D. macarellus*	75	**100.00**	**KU943796.1**
RP2	*D. macarellus*	73	**100.00**	**KU943796.1**
RP3	*D. macarellus*	73	**100.00**	**KU943796.1**
RP4	*D. macarellus*	81	**99.83**	**KY570722.1**
RP5	*D. macarellus*	84	**100.00**	**MH085884.1**
RP6	*D. macarellus*	85	**100.00**	**MH638719.1**
RP7	*D. macarellus*	74	**100.00**	**KU943796.1**
RP8	*D. macarellus*	83	**100.00**	**MH638719.1**
RP9	*D. macrosoma*	74	**99.82**	**KU943769.1**
RP10	*D. macrosoma*	69	**100.00**	**KU943769.1**
RP11	*D. macrosoma*	75	**100.00**	**KU943769.1**
RP12	*D. macrosoma*	73	**100.00**	**KU943769.1**
RP13	*D. macrosoma*	74	**100.00**	**KU943769.1**
RP14	*D. macrosoma*	81	**100.00**	**MH638663.1**
RP15	*D. macrosoma*	90	**100.00**	**HQ560948.1**
RP16	*D. macrosoma*	73	**100.00**	**KU943769.1**
RP17	*D. russelli*	74	**99.82**	**KU943718.1**
RP18	*D. russelli*	75	**99.82**	**KU943718.1**
RP19	*D. russelli*	75	**99.82**	**KU943718.1**
RP20	*D. russelli*	79	**99.83**	**JQ681458.1**
RP21	*D. kurroides*	86	**99.84**	**JN312965.1**
RP22	*D. kurroides*	83	**99.67**	**JN312965.1**
RP23	*D. kurroides*	85	**99.23**	**JN312965.1**
RP24	*D. kurroides*	87	**99.54**	**JN312965.1**

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All specimens have a DNA barcode (DNA fragments from the total DNA fragments of COI gene analyzed by the BLAST program) around 700-800bp in length. Savolainen et al. 2005 explained that the ideal DNA barcode is a DNA fragment with a short and uniform sequence with a length of 400-800bp that can be quickly produced and used in the identificaiton. Every specimen has different levels of similarity in its nucleotide base sequence (identified (%) based on the DNA fragment from the COI gene being analyzed. Song et al. (2008) proposed that species identification using DNA barcoding should be based on sequence similarity. Greater percentage value of up to 100% shows more accurate the species identification. Based on the "identified" presentation, all specimens on the four species of scad have been correctly identified based on the Gene Bank with a percentage of over 99%. According to Hebert et al. 2003, species with a genetic distance of >3% are considered interspecific species.

The Validation of The Morphological Identification Using Molecular Identification

There is a difference in the number of species of the 24 specimens of scad morphologically identified when compared with molecular identification (DNA barcoding) (Table 3). Six species of scad are identified based on their morphological characteristics; *D. macarellus* (ID number RP1-RP4), *D.macarellus** (ID number RP5-RP8), *D. macrosoma* (ID number RP9-RP12), *D. macrosoma** (ID number RP13-RP16), *D. russelli** (ID number RP17-RP20) and *D. kurroides* (ID number RP21-RP24). Furthermore, seen from the DNA barcoding, only four species of scad were successfully identified: *D. macarellus* (ID number RP1-RP8), *D. macrosoma* (RP9-RP16), *D. russelli* (ID number RP17-RP20), and *D. kurroides* (ID number RP21-RP24).

In the beginning, Scad with ID RP1-RP8 consisted of two species: *D. macarelllus* dan *D. macarellus**. However, in DNA barcoding, the Scad with ID RP1-RP8 appeared to consist of only *D. macarellus.* Similarly, Scad with ID RP11-R20 was initially identified consisting of 2 species based on the morphological analysis: *D. macrosoma* dan *D. macrosoma**. However, the DNA barcoding confirmed that there is only one species: *D.* macrosoma. Likewise, *D. russelli** (ID RP21-RP25) which had been falsely identified, appeared to have four Scad species: *D. macarellus*, *D. macrosoma* dan *D. russelli.* dan *D. kurroides.*

Table 3. The comparison of scads species based on the morphological identification with the molecular identification.

ID	Species	
	Morphological identification	Molecular identification
RP1-RP4	*D. macarellus*	*D. macarellus*
RP5-RP8	*D. macarellus**	*D. macarellus*
RP9-RP12	*D. macrosoma*	*D. macrosoma*
/RP13-RP16	*D. macrosoma**	*D. macrosoma*
RP17-RP20	*D. russelli**	*D. russelli*
RP21-RP24	*D. kurroides*	*D. kurroides*
Number of Species	4 *suspected to be other species	4

After being validated using the results of DNA barcoding, three species of scad which were originally still "suspected" based on the morphological identification; *D. macarellus**, *D. macrosoma**, and *D. russelli**, with all of the species being identified as *D. macarellus*, *D. macrosoma* dan *D. russelli*. It shows that differences in morphological characteristics of every individual vary in every Scad species yet they do not necessarily affect the genetic differences. Zhang & Hanner (2011) proposed that for fish, a large part of intraspecific diversity or interspecific overlapping in the identification process affects the accuracy of the identification. According to Bohlke & Chaplin (1993), fish in the *Carangidae* family, including scad, often show significant changes in morphology and pigmentation throughout growth, leading to incorrect identification.

The overlapping characteristics and significant genetic variation between *D. macrosoma* and *D. macarellus* represent a weakness in characteristic-based identification, as seen in Table 1 & Figure 2. Inaccuracy of fish species identification using morphological methods is common since identification keys often require a high level of expertise (Hebert et al., 2003). Several experts mentioned some factors that may interfere with the identification, including the large number of morphological characters used in identification and the variation in these characters due to geographical differences, sex differences, and character differences in each life phase (Heemstra & Randall, 1993; Blaxter, 2006; DeSalle, 2006; Victor et al., 2009) and the existence of Cryptic species (morphologically similar but

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genetically different) (Hubert et al., 2012). In much research, the molecular approach has been used in the scad identification process at the species level. Furthermore, DNA Barcoding has been regarded as a suitable complementary taxonomic tool in quicker and accurate species identification.

Genetic and Phylogenetic Distance

Based on the results of molecular validation (DNA barcoding), four species were identified from 24 fish specimens of Momar. After the editing, 24 COI gene sequences from 24 Momar specimens resulted in a sequence length of 635 bp, which genus and species are shown in Table 4.

Table 4. Genetic differences (percentage of K2P distance) within taxonomic levels

% K2P Genetic Distances			
Comparison Within	*Min*	*Max*	*Mean*
Genus (Interspecific)	6.12	13.29	10.18
Species (Intraspecific)	0.00	1.75	0.54

The intraspecific K2P genetic of COI genes ranges from 0.00 to 1.75% (mean 0.54%); with the smallest genetic distance (0.00%) in the species *D. macarellus* and *D. macrosoma* and the most considerable genetic distance (1.75%) between specimens in the species *Decapterus russelli* (Table 5). The interspecific K2P genetic range of COI genes is between 6.12-13.29% (mean 10.18%), with the smallest genetic distance (6.12%) between specimens from *D. macarellus* species and specimens from *D. macrosoma* species and the most significant genetic distance (13.29%) between specimens from *D. russelli* species and specimens from *D. kurroides* species (Table 6). The average of interspecific K2P genetic distance, of10.18%, is significantly greater than the average of intraspecific K2P genetic distance or about 19 times the average of intraspecific K2P genetic distance. The mean inter-specific genetic distance (10.18%) is significantly higher than the average intra-specific genetic distance (0.54%), indicating that the genetic characteristics between specimens from one species to another have are quite large than the genetic characteristics between specimens of the same species.

Table 5. Genetic differences (percentage of K2P distance) within species (intraspecific)

% K2P Genetic Distances							
Species	*Min*	*Max*	*Mean*	*Nearest Neighbor*		*Farthest Neighbor*	
				Species	*Mean*	*Species*	*Mean*
D. macarellus	0.00	0.79	0.29	*D. macrosoma*	6.42	*D. kurroides*	10.03
D. macrosoma	0.00	0.95	0.42	*D. macarellus*	6.42	*D. russelli*	11.22
D. russelli	0.47	1.75	1.03	*D. macarellus*	10.01	*D. kurroides*	12.75
D. kurroides	0.16	0.63	0.42	*D. macarellus*	10.03	*D. russelli*	12.75

Table 6. Genetic differences (percentage of K2P distance) within genus (interspecific)

Comparison Within Genus	*% K2P Genetic Distances*		
	Min	*Max*	*Mean*
D. macarellus vs D. macrosoma	6.12	7.15	6.42
D. macarellus vs D. russelli	9.31	10.76	10.01
D. macarellus vs D. kurroides	9.56	10.46	10.03
D. macrosoma vs D. russelli	10.59	11.89	11.22
D. macrosoma vs D. kurroides	10.12	11.24	10.67
D. russelli vs D. kurroides	12.17	13.29	12.75

A phylogenetic tree (NJ) was created based on 24 DNA barcode sequences (Figure 4) which group similar specimens forms of monophyletic clusters (from the same ancestor) that are separated from each other, with the support of bootstrap values of 100%. Within the monophyletic group formed, some subgroups have bootstrap values support ranging between 40-64%. The most negligible (40%) and the largest (64%) bootstrap support was found in the subgroup nodes of the *D. ruselli* species (Figure 4 (b)).

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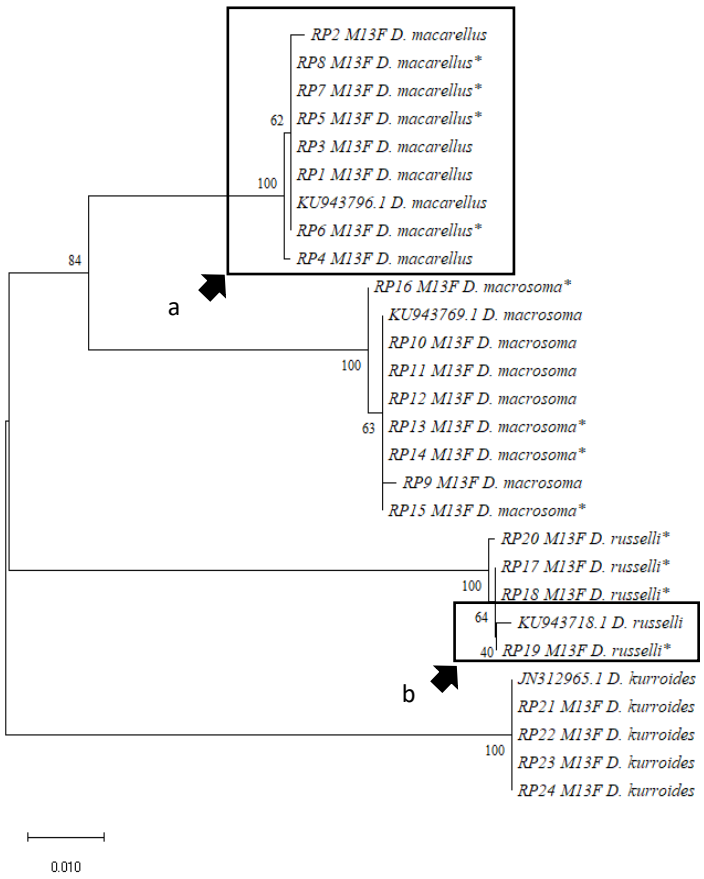

Figure 4. A phylogenetic tree created based on 24 DNA barcode sequences yielded a) a monophyletic group, b) a subgroup within a monophyletic group.

The mitochondrial COI gene is commonly used as a species barcode due to its distinctive pattern of genetic variation between species (Hebert et al., 2003b). The K2P model is employed in this study because it offers consistency and facilitates comparison with other studies.

Hebert et al. (2003a) suggested that DNA sequences would be more similar within species (intra-specific) than between species (inter-specific). The observed range of intra-specific genetic distance, from 0.00% to 1.75%, indicates that all specimens belong to the same four identified species. According to Hebert et al. (2003a), species with a genetic distance greater than 3% are classified as inter-specific.

Specimens were grouped based on genetic similarity, consistently resulting in the same pattern across 1000 repetitions. Identical specimens formed a monophyletic group with a 100% bootstrap value, demonstrating that COI-based DNA barcoding can accurately identify scad fish species (Ran et al., 2020). The grouping pattern remained consistent.

The formation of subgroups among similar specimens is due to their high genetic similarity. Slight differences in genetic distance between similar specimens (intra-specific) lead to the creation of subgroups within the monophyletic group (Figure 4b). Increased branching or the number of subgroups within a monophyletic group indicates higher genetic variation among specimens within a species.

4. Conclusion

Morphological identification based on morphometric and meristic calculations initially indicated six species of scads in Ambon's waters, with three of these being uncertain and suspected to be other species. However, DNA barcoding confirmed the presence of only four species: *Decapterus macarellus, D. macrosoma, D. russelli, and D. kurroides.*

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