Description and identification of tuna larvae based on genetic and morphological analysis

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\textbf{Abstract}. Biological information of tuna larvae is essential for understanding spawning ground and time. However, morphological identification of fish larvae is difficult and questionable. Meanwhile, genetic identification is useful and helpful to confirm the morphological identification result. This study purpose is to confirm a morphological identification result with genetic identification and determine the tuna species exactly. Specimens were obtained from a survey conducted in the Banda Sea in October 2018. Samples were collected by a bongo net towed obliquely. The result of morphological identification is showed that the larvae having 37 to 42 myomeres, preopercular spine developed, moderate in-depth, moderate to large head and eyes identified as \textit{Thunnini} larva. The morphological result is supported by the genetic result, even though the larvae are not from the genus of \textit{Thunnus}. The samples were identified as \textit{Katsuwonus pelamis}, \textit{Euthynnus affinis}, and \textit{Petenia splendida}. The intraspecific pairwise genetic distance for each species of \textit{K. pelamis}, \textit{E. affinis}, and \textit{P. splendida} is 4.4\%; 0.5\%; and 22.3\%, respectively. Meanwhile, the average pairwise genetic distance between the three species ranges from 16.2\% to 33.3\%.

\textbf{Keywords}: Tuna larvae, Morphological identification, Genetic identification

1. Introduction

Tuna is one of the important commodities in Indonesia where the country contributes around 14\%  (720,500 tons) of the global tuna catch \cite{1}. It consists of four main oceanic tuna namely yellowfin (\textit{Thunnus albacares}), bigeye (\textit{T. obesus}), albacore (\textit{T. alalunga}) and skipjack tuna (\textit{Katsuwonus pelamis}). Those commercial fishes are well-known as highly migratory species crossing different seas. The fisheries management in Indonesia aiming to sustain the tuna stock become a spotlight since the progress in combating illegal, unreported and unregulated (IUU)
fishing, and also the issuance of the decree to protect and preserve the tuna resources area, such as in the Banda Sea [2].

One way to conserve the tuna stock is to understand the biological information of tuna larvae, including understanding its life stage, spawning ground, and spawning time [3, 4]. Unfortunately, the knowledge of tuna larvae particularly in Indonesian waters is still lacking. A review study on tuna larvae and reproductive biology found that there is a big gap on the scientific knowledge in supporting the regulation of temporary closure of the area in the Banda Sea that is intended to conserve yellowfin tuna stock [5].

Furthermore, there was only one publication studying tuna larvae using the morphological approach to identify the species. In fact, the identification of the tuna larvae based on the morphological character is difficult and lead to misidentification or bias, and often questionable [6, 7, 8, 9]. Tuna larvae belonged to the tribe of Thunnini (family of Scombridae) are difficult to be distinguished from other species in the same family. The challenge starts with the preserving method in which the larval sample is fragile and prone to damage. The morphological identification of larvae is more complicated due to its size. However, the main features such as meristic (myomeres), morphology (shape of the first dorsal fin and preopercular spine) and morphometric (snout length, body length, eye diameter, head length and trunk) can be used as the characters for identification [6, 10, 11]. Moreover, morphological identification of larvae suggested only conducted up to the family level; and it requires a higher and advanced skill of identification to identify it into the species level [8, 12].

As the development of the molecular genetic study, it is proven that the molecular can be used as a method to support morphological identification [13, 14]. However, the strong support of the genetic method is in the availability of the genetic database. Therefore, in the absence of genetic data of several species, the best approach for identification is to combine both morphological and genetic methods. This study aims to identify the species of tuna larvae using both morphological and genetic methods.

2. Materials and Methods
2.1. Sample Collection
Specimens used in this research were obtained from a survey conducted in the Banda Sea in October 2018. Samples were collected using a bongo net (0.5 m in diameter with mesh size 500 µm) from 6 sampling stations. The bongo net was towed obliquely through the water from 120 meters to surface for about 6 minutes, at a 2 knots speed. Samples were preserved with 5% buffered formaldehyde (in seawater) on-board and labelled for laboratory work. In the laboratory, fish larvae were sorted out from other organisms and stored in small vials in 70% alcohol.

2.2. Morphological Observation
The identification of larvae is conducted into the tribe level – between family and genus. Tuna larvae were observed under a stereomicroscope Nikon SMZ1270i and photographed using a camera Nikon D5500, which integrated on the microscope. Specimens collected were identified using various morphological features, meristic and morphometric, and identify which to the lowest possible taxon using available literature [10, 11, 15, 16, 17].
2.3. Genetic Analysis

Small tissue fragment from each sample was extracted using a 10% chelex solution in order to get its genomic DNA [18]. The control region (CR) mitochondrial DNA (mtDNA) fragment was amplified using the polymerase chain reaction (PCR) method with the published CRK-CRE primers [19]. The PCR reaction was carried out in 25 μL volumes, using 1 μL of the template. Each reaction included 2.5 μL 10x PCR buffer (Applied Biosystem), 2.5 μL 10 mM dNTPs, 1.25 μL of each primer at 10 mM, 2 μL 25 mM MgCl₂ solution, 0.125 μL AmplyTaq Red™ (Applied Biosystem), and 14.5 μL ddH₂O. The thermocycling profile included an initial denaturation of 94°C for 15s, 38 cycles of 94°C for 30s, 50°C for 30s, and 72°C for 45s, with a final extension of 72°C for 5 min. PCR products were visualized on 1% agarose gels stained with Biotium® gel red stain, and sequence using both primers using the Big Dye Chain Termination.

Forward and reverse sequences were proofread using MEGA5 [20] and then aligned using CLUSTALW, with subsequent alignment by eye. Species identity for each sample was determined by comparing the sequences data with the GenBank database (www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool), enforcing a sequence homology threshold of ≥97%. Phylogenetic analysis was then performed using Neighbour-Joining [21], with 1,000 bootstrap replication. Genetic distance between individual sequences calculated using Kimura 2-parameter methods [22]. Sequences of *E. affinis* (AP012946) *K. pelamis* (KM094133, KM261666, KM094145, KM605252, KM094135), and *P. splendida* (KJ914664) were added to the phylogenetic analysis for species comparison.

3. Results and Discussion

3.1. Morphological Observations

Samples have 37 to 42 myomeres, preopercular spine developed, moderate in-depth with proportion body depth to body length between 23.88% and 28.27%, moderate to large head and eyes, which then identified as a tuna larva. However, the morphometric of the sample (BIO.SUB34.01.003_ST1) cannot be measured due to the curved body shape (Table 1). As one of the species in the family of Scombridae, tuna is generally elongate to moderate, laterally compressed, and most are abruptly deeper in head and gut than in the tail. The inconspicuous gad bladder is located just above the apex of the gut [11, 15, 16].

| No | ID        | Preopercular Spine | Myomeres | Body Length (mm) | Depth (mm) | Proportion BD to BL (%) | Category | Head Length (mm) | Proportion HL to BL (%) | Category | Head Diameter (mm) | Proportion ED to HL (%) | Category | Snout Length (mm) | Trunk Width (mm) |
|----|-----------|---------------------|----------|------------------|------------|--------------------------|----------|------------------|--------------------------|----------|-------------------|--------------------------|----------|------------------|---------------------|
| 1  | BIO.SUB34.01.001_ST1 | Developed          | 42       | 3.80             | 1.00       | 26.32                    | Moderate | 1.25             | 32.89                    | Moderate | 0.4               | 32.00                    | Moderate | 0.55             | 0.7                 |
| 2  | BIO.SUB34.01.002_ST1 | Developed          | 41       | 1.95             | 0.52       | 26.67                    | Moderate | 0.66             | 33.85                    | Large    | 0.25              | 37.88                    | Large    | 0.23             | 0.38                |
| 3  | BIO.SUB34.01.003_ST1 | Developed          | 39       | -                | -          | -                        | Moderate | -                | -                        | -        | -                 | -                        | -        | -                | -                   |
| 4  | BIO.SUB34.01.004_ST1 | Developed          | 37       | 4.57             | 1.25       | 27.35                    | Moderate | 1.58             | 34.57                    | Large    | 0.48              | 30.30                    | Moderate | 0.68             | 0.85                |
| 5  | BIO.SUB34.01.002_ST2 | Developed          | 39       | 4.23             | 1.01       | 23.88                    | Moderate | 1.32             | 31.21                    | Moderate | 0.43              | 32.58                    | Moderate | 0.54             | 0.69                |
| 6  | BIO.SUB34.01.002_ST2 | Developed          | 40       | 4.28             | 1.21       | 28.27                    | Moderate | 1.39             | 32.48                    | Moderate | 0.45              | 32.17                    | Moderate | 0.52             | 0.67                |
| 7  | BIO.SUB34.01.001_ST1 | Developed          | 41       | 3.78             | 0.97       | 25.66                    | Moderate | 1.22             | 32.28                    | Moderate | 0.41              | 33.61                    | Large    | 0.53             | 0.65                |

BD: Body Depth   BL: Body Length   HL: Head Length   ED: Eye Diameter

Moreover, Scombrids are characterized by large heads and eyes (except that in some pre-flexion larvae with rounded heads, it may be moderate), head spination and triangular (except the Scombrini), myomere number (31-64), pigmentation pattern, triangular, compact and coiled...
gut [10, 11, 23]. Meanwhile, in the study conducted by [6, 10, 11] indicated that all tuna larvae possess the following characteristics: triangular and relatively large head, eyes, and mouth, relatively short pointed snout, relatively short body (37-42 myomeres), triangular abdominal cavity, very sparse and weakly developed body pigments, developed pre-opercular and post-temporal spines, and the absent of supraoccipital spine (except for *Euthynnus*).

![Figure 1. Photograph of fish larvae identified as tuna larvae.](image)

The different number of myomere is useful to separate *Katsuwonus* and *Euthynnus* from other tunas [10]. However, the number of fin rays and spines are not useful for species identification of *Thunnus* group because all species are similar in this respect. The shape of the first dorsal fin, when completely formed, is useful to distinguish late larval stages of
Katsuwonus, Euthynnus, and Auxis from those of Thunnus. Pre-opercular spines are not one of the reliable features for identification.

Despite its disadvantages, morphometric has been used as one of the methods to described species by using the relations of body depth to standard length, snout length to head length, and snout length to orbit diameter. One of the reasons for the measurement data to be biased is because the larvae may shrink in the preservatives. The degree of shrinkage may vary in different preservative types and time duration. Within the time of samples preservation, the distortion of the larvae body also cannot be controlled [6].

Meanwhile, the black pigment pattern has been the most widely used and accepted character in identifying tuna larvae. There are variations and changes in black pigment patterns on tuna larvae due to growth, but only in a certain area of the body. These patterns have been found to be consistent for identification purposes, which is particularly true for pigment patterns on the first dorsal fin, posterior half of the trunk, forebrain, and tips of both jaws [6].

The study of [8] found that the average accuracy rates of morphological identification were quite low, i.e. 80.1% for family level, 41.1% for genus level, and 13.5% for species level. Thus, the best identification for tuna larvae is up to the family level, and it is extremely hard to conduct up to the genus or species level.

To address the insufficient of morphological identification, the Cytochrome c oxidase subunit I (COI) gene is a potential technique to identify tuna larva until species level and COI itself has widely applied in species identification of other fish species [9, 14, 26, 27, 28]. Comparing to the morphological method, DNA barcoding has some advantages, one of those is efficient and accurate for species identification [27].

3.2. Genetic observations
Genetic identification was performed using 523 bp of mitochondrial control region locus, with 58 parsimony informative sites within this region. Genetic result identification of 7 fish larvae using comparison with the GenBank database (BLAST) was shown in Table 2, with the homology threshold of >97%, except for the samples identified as P. splendida (homology of 83%). The samples were identified as K. pelamis, E. affinis, and P. splendida.

| No | ID                   | BLAST Identification           | Query cover (%) | Identical (%) |
|----|----------------------|--------------------------------|-----------------|---------------|
| 1  | BIO.SUB34.01.001_ST1 | Euthynnus affinis              | 100             | 99            |
| 2  | BIO.SUB34.01.002_ST1 | Katsuwonus pelamis             | 100             | 98            |
| 3  | BIO.SUB34.01.003_ST1 | Katsuwonus pelamis             | 100             | 98            |
| 4  | BIO.SUB34.01.004_ST1 | Petenia splendida              | 51              | 83            |
| 5  | BIO.SUB34.02.001_ST2 | Katsuwonus pelamis             | 100             | 97            |
| 6  | BIO.SUB34.02.002_ST2 | Katsuwonus pelamis             | 100             | 97            |
| 7  | BIO.SUB34.04.001_ST4 | Katsuwonus pelamis             | 99              | 98            |

Species identification result using phylogenetic tree analysis was shown in Figure 2. The result of the phylogenetic tree was strongly supported by the BLAST result, which also indicated 3 different species from the samples collected.
Figure 2. Phylogenetic tree generated from the control region mtDNA data from 7 samples of fish larvae and GenBank database sequence. The number on the nodes indicated the bootstrap number.

Based on the comparison with the database sequence, the intraspecific pairwise genetic distance for each species of *K. pelamis*, *E. affinis*, and *P. splendida* are 4.4%; 0.5%; and 22.3%, respectively. Meanwhile, the average pairwise genetic distance (Table 3) between the 3 species ranges from 16.2-33.3%.

**Table 3.** Average pairwise genetic distance between 3 species.

| Species                  | 1     | 2     |
|--------------------------|-------|-------|
| *Euthynnus_affinis*      | 1     |       |
| *Katsuwonus_pelamis*     | 2     | 0.162 |
| *Petenia_splendida*     | 3     | 0.325 | 0.333 |

Within this result, we resolved the identification of 6 samples using genetic methods and identify the samples as the tuna group of genera *Euthynnus* and *Katsuwonus*. However, one of the samples indicated as BIO.SUB34.001.004_ST1 were not fully resolved, due to the low percentage of identity and query cover when compared to the database. Although the samples were matched as *P. splendida* in the GenBank database, the low value of bootstrap and high intraspecific distance may indicate that this sample was not strongly supported as the species of *P. splendida*. Therefore, although DNA barcoding is one of the useful methods to confirm the accuracy of traditional fish identification, a complete database is very crucial for identification using genetic data.

As the addition of this result, further research and in-depth use of morphological characters are needed to answer the question about the species identification for this particular sample, and also the importance of the addition of more loci for better support on the genetic data analysis.
4. Conclusion

The study shows the complexity of the morphological identification of tuna larvae. Despite observing some key features of the larvae body such as myomere, body shape and eye diameter could be beneficial, the misleading remains possible due to some circumstances of the larvae sample i.e. preservatives method and time duration. Therefore, we suggest to doing tuna larval identification in precautionary where it is better to key only to the family and not to the genus or species stage. From this study, we conclude that genetic identification is still one of the best ways to confirm species identification. Nevertheless, our study will add and complement the information on the genetic and biogeographic public databases of Scombridae, particularly on tuna species. The study findings will assist the future studies in strengthening the policy of the Banda Sea temporary closure area.

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6. References

[1] Food and Agriculture Organization (FAO) 2016 Commodity update tuna by globefish (Rome: FAO–Fisheries and Aquaculture Policy and Economics Division) p 51

[2] Ministry of Marine Affairs and Fisheries (MMAF) 2015 Ministry Regulation No.04/2015 concerning prohibition of fishing activities in the republic of Indonesia fishery management area (WPP RI) 714 (Jakarta: Ministry of Marine Affairs and Fisheries–Republic of Indonesia) p 4

[3] Dulcic J 1998 Infection of sardine eggs by a parasitic dinoflagellate Ichthyodinium chabelardi Hollande and Cachon, 1952 in Croatian waters Annales: Analı́ za Istrske in Mediteranske študije. Series Historia Naturalis 13 15-18

[4] Di Natale A 2017 Scientific needs for a better understanding of the atlantic bluefin tuna (Thunnus thynnus) spawning areas using larval surveys Collect. Vol. Sci. Pap. ICCAT 73 (7) 2255-2279

[5] Satrioaie W N, Suyadi, Syahailatua A and Wouthuyzen S 2018 The importance of the Banda Sea for tuna conservation area: A review of studies on the biology and the ecology of tuna IOP Conf. Ser.: Earth Environ. Sci. 184 1 p 012004

[6] Matsumoto W M, Ahlstrom E H, Jones S, Klawe W L, Richards W J and Ueyanagi S 1972 On the clarification of larval tuna identification Fish. Bull. 70 1-12

[7] Chow S, Nohara K, Tanabe T, Itoh T, Tsuji S, Nishikawa Y, Ueyanagi S and Uchikawa K 2003 Genetic and morphological identification of larval and small juvenile tunas (Pisces: Scombridae) caught by a mid-water trawl in the western Pacific Bull. Fish. Res. Agen. 8 1-14
[8] Ko H-L, Wang Y-T, Chiu T-S, Lee M-A, Leu M-Y, Chang K-Z, Chen W-Y and Shao K-T 2013 Evaluating the accuracy of morphological identification of larval fishes by applying DNA barcoding PLoS ONE 8 1
[9] Puncher G N, Alemany F, Arrizabalaga H, Cariani A and Tinti F 2015 Misidentification of bluefin tuna larvae: a call for caution and taxonomic reform Rev. Fish Biol. Fisheries 25 485-502
[10] Nishikawa Y and Rimmer DW 1987 Identification of larval tunas, billfishes and other scombroid fishes (suborder Scombroidei): an illustrated guide, series report Commonwealth Scientific and Industrial Research Organisation (Australia) Marine Laboratories no. 186 (Melbourne: CSIRO) p 25
[11] Leis J M and Carson-Ewart 2004 The larvae of Indo-Pacific coastal fishes: an identification guide to marine fish larvae 2nd ed (Leiden; Boston: Brill) p 850
[12] Rodriguez A E, Martinez J C, Garcia J R and Ramirez J T 2007 Description of a distinct Snapper larvae and species identification based on mitochondrial DNA analyses GCFI 58 168-172
[13] Baldwin C, Mounts J H, Smith D G and Weigt L A 2009 Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with comments on identification of adult Phaeoptyx Zootaxa 2008 1-22
[14] Teletchea F 2009 Molecular identification methods of fish species: reassessment and possible applications Rev. Fish Biol. Fisheries 19 265
[15] Neira F J, Miskiewicz A G and Trnski T 1998 Larvae of temperate Australian fishes: laboratory guide for larval fish identification (Nedlands: University of Western Australia Press) p 474
[16] Kendall A W 2011 Identification of eggs and larvae of marine fishes (Kanagawa: Tokai University Press) p 379
[17] Izumi K, Tomoji I, Akio H, Shigeo T, Yosuke O and Satoshi M 2014 日本産稚魚図鑑 = An atlas of early stage fishes in Japan: 2nd ed (Kanagawa: Tokai University Press) pp 977-1641
[18] Walsh P S, Metzger DA and Higushi R 1991 Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material BioTechniques 10 506-513
[19] Lee W J, Conroy J, Howell W H and Kocher T D 1995 Structure and evolution of teleost mitochondrial control regions J. Mol. Evol. 41 54-66
[20] Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods Mol. Biol. Evol. 28 2731-2739
[21] Saitou N and Nei M 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees Mol. Biol. Evol. 4 406-425
[22] Kimura M 1980 A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences J. Mol. Evol. 16 111-120
[23] Mwaluma J M, Kaunda-Arara B and Strydom N A 2014 A guide to commonly occurring larval stages of fishes in Kenyan Coastal Waters, WIOMSA Books Series No. 12 (Dar es Salaam: Jamana Printers Limited) p 96 http://www.wiomsa.org/
[24] Zhang J and Hanner R 2012 Molecular approach to the identification of fish in the South China Sea PLoS ONE 7 1
[25] Bingpeng X, Heshan L, Zhilan Z, Chunguang W, Yanguo W, and Jianjun W 2018 DNA barcoding for identification of fish species in the Taiwan Strait *PLoS ONE* **13** 6

[26] Hulley E N, Taylor N D J, Zarnke A M, Somers C M, Manzon R G, Wilson J Y, and Boreham D R 2018 DNA barcoding vs. morphological identification of larval fish and embryos in Lake Huron: Advantages to a molecular approach *J. Great Lakes Res.* **44** 1110-1116