Molecular Identification of Fish Larvae from East Plawangan of Segara Anakan, Cilacap, Central Java, Indonesia

Agus Nuryanto, Hendro Pramono, Moh Husein Sastranegara

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Abstract
Correct identification of fish larvae from East Plawangan is very difficult to be done due to undefined of their morphology, while correct name is an important information for management of that area as spawning and nursery ground. Therefore, so far no scientific report on what species that utilize East Plawangan as their spawning and nursery ground. Cytochrome c oxidase 1 gene provide a precise tool for correct larvae identification. This study aims to test the accuracy of the cytochrome c oxidase 1 gene on fish larvae identification. The COI gene were amplified and sequenced. The homology of sequences were checked using Basic Local Alignment Search Tool (BLAST). Genetic distances were calculated with the help of MEGA 6 software. Taxonomic tree was reconstructed using neighbor-joining and maximum parsimony methods with 1000 bootstraps repeats. A sum of 24 morphotypes were identified, indicating high diversity of fish that utilize East Plawangan as their spawning and nursery ground. BLAST result showed that seven morphotypes were convincingly identified into species level, while three remaining morphotypes could only be identified at generic level. Taxonomic tree shows clear discrimination among morphotypes. This proved that cytochrome c oxidase 1 gene showed high accuracy on larval species discrimination.

How to Cite

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INTRODUCTION

Segara Anakan lagoon is a brackish water estuary located in South Coast of Cilacap Regency, Central Java. This lagoon is connected to Indian ocean by areas so-called East Plawangan and West Plawangan, in the east part and in the west part of the lagoon respectively (Manez, 2010). The size of Segara Anakan Lagoon has been reduced from approximately of 4,000 ha (Naamin, 1991) become 2,200 ha in a recent year (Manez, 2010). This is occurred due to human activities (Ardli, 2007).

Segara Anakan Lagoon is surrounded by mangrove forest. This provides various micro-habitat either for aquatic, terrestrial, or arboreal organisms. Management is vital to ensure the sustainability of ecological function of Segara Anakan Areas, including the lagoon. An effective management policy will only be achieved if it is arranged based on a support from various scientific information, including data on fish species that utilize Segara Anakan Lagoon as the spawning ground and nursery ground. Previous studies from Romimohtarto et al. (1991) and Ardli (2007) had reported that various fish species used Segara Anakan Lagoon as the feeding ground and nursery ground before the larvae are transported to open sea water through the tide current. It has been reported that a total of 45 fish species were utilized Segara Anakan Lagoon as living ground (White et al., 1989). However, no scientific report available so far on fish species utilizing Segara Anakan Lagoon as their spawning ground and nursery ground.

Traditional taxonomic study use morphological characters for species identification. However, such study can not be applied on larval stages because its morphological characters are still unstable (Leis and Carson-Ewart, 2004). In one hand, larval morphology might alter correspond to their life stages (Ko et al., 2013). In other hand, morphological characters are also shared by larvae of different species (Victor et al., 2009). Those both conditions might cause miss-identification of the larva (Ko et al., 2003). In this case, DNA barcoding offers a precise method to solve the problem with no doubt (Bucklin et al., 2003; Hebert et al., 2003a; von der Heyden et al., 2014). Moreover, Frankham (2003) proved that molecular characters are helpful tools for species identification with higher accuracy than with previous methods. Hebert et al. (2003b) proposed that cytochrome c oxidase 1 gene as a reliable barcode for species identification. Previous studies have proved that the COI gene is strong enough for fish species discrimination (Ward et al., 2005; Sachithanandam et al., 2012; Dor, 2012; Nuryanto and Pramono, 2015).

Using this technique, species can be discriminated based on their genetic distances with the value ranges from 2 to 3 % (Hebert et al., 2003b) or ten-fold in bird species (Hebert et al., 2004). According to Barber et al. (2002) crustacean species can be separated by the genetic distance of 2%, while Nuryanto et al. (2007) reported that genetic differences among Tridacna species ranging from 5% to19%.

Here we applied DNA barcoding technique to identify fish larvae collected from East Plawangan, Segara Anakan Lagoon. Therefore the aim of this study was to test the accuracy of cytochrome c oxidase 1 on fish larvae identification into species level.

METHODS

Sample collection

Fish larvae were collected randomly using larvae nets at East Plawangan areas of Segara Anakan Lagoon, including the mouth areas of East Plawangan, downstream of Sapuregel River areas and Donan River across Pertamina (Figure 1). Larvae sampling was performed by tighten the larvae nets on the back part of the boat while driving with the speed of approximately of 10 knots. The larvae nets was raised every ten minutes to collect the larvae. This sampling techniques were done with driving direction from the centre of East Plawangan to Sapuregel River areas and back, from the centre of East Plawangan to Donan River areas and back, and also from the centre of East Plawangan to the mouth of East Plawangan areas and back (Figure 1). Sampling efforts at each direction were repeated four times. The samples were preserved in absolut ethanol 96 % and stored in the freezer at the temperature of 4 °C until DNA isolation.

DNA isolation

Total genomic DNA was isolated using Thermoscientific DNA extraction kits following the protocol from the company (https://www.thermofisher.com).

DNA amplification

The COI gene fragments were amplified using a pair of universal primers LCO 1490: 5'-GGT CAA CCA ATC ATA AAG ATA TTG G-3' and HCO 2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer et al., 1994). The PCR reaction was then used in a to-
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A total of 242 individuals of larvae were collected during the filed trips. Morphological observation under stereo microscope placed the 242 individuals of larvae into 24 fish morphotypes. The data proved that East Plawangan of Segara Anakan inhabited by high diversity of fish, indicating the important of East Plawangan as the spawning and nursery ground. According to NCDENR (2006) ecosystem inhabited by more than 19 fish species indicated high fish species diversity. The important of East Plawangan as the spawning and nursery ground is raised by the presence of larvae from various organism, such as shrimps, crabs, and jelly fish.

Instead of using adult individuals, in this study we used fish larvae for species identification. The reason behind this selection was due to that we need information about fish species that utilize East Plawangan of Segara Anakan as the spawning and nursery ground. Precise data on those information would be very difficult to be obtained if we use adult individuals. In one hand, it is because not all adult individuals live in East Plawangan will spawn and nurse their larvae in that areas. In other hand, it is also well known that not all adult individuals of the larvae which are found in East Plawangan live on that areas. For instance, we can obtain larval stages of Anguilla spp. in East Plawangan but their adult individuals can only be found either in the upper-stream of a river or in the deep sea during spawned and the larvae will move to East Plawangan in order to complete their life cycles. Therefore, precise information about the species that use East Plawangan as their spawning and nursery ground can only be obtain through larval identification.

However, it is very difficult to identify fish larvae morphologically due to undefined their morphological characters. Molecular identification or DNA barcoding is only method to precisely identify fish larvae. According to Shao et al. (2002) molecular identification provide precise tool for larvae identification.

So far, sequencing of the COI fragment was successfully sequenced from ten morphotypes. In BLAST, the morphotypes showed the different degree of sequence similarity with COI gene in the NCBI nucleotide database, with range values from 90% to 99%. This result indicated that some morphotypes can be identified convincingly into
species level, while some other morphotypes can only be identified into generic level. According to BLAST result, seven out of ten morphotypes can be identified to species level with the homology of sequences of 98% to 99% to the COI sequences of certain species available in GeneBank. The three remaining sequences could only be identified as generic level since they have only 90% to 94% sequences similarity to the available COI database in NCBI. Our finding is congruence to Peg et al. (2006) who reported that individuals have sequence similarity of 97% to 99% can be considered as a single species.

Two morphotypes (A1 and D2) showed similar values of sequence similarity to COI sequence of single species available in the NCBI database. Different morphology among A1 and D2 could be due to that both individuals are at different life stages on their life cycles although they are belong to the same species. According to Ko et al. (2013) two individuals of larvae from single species but at different life stages can be identified as two different species because they have different morphology. Therefore, it was reasonable if morphologically we identified as A1 and D2 morphotypes but both morphotypes are belong to a single genetic species. This result proved the powerful of the COI gene identification over morphological-based identification. According to Shao et al. (2002), molecular identification can precisely identified fish species from eggs and it can be adopted for fish larvae identification.

Morphotype C1 was identified as *Secutor megalolepis* (98% similarity to species in GeneBank with accession number DQ648454.1); morphotype D1 was identified as *Stolephorus indicus* (99% sequence similarity to available data with accession number KU935459); morphotype D3 was identified as *Favonigobius gymnauchen* (98% sequence similarity to accession number KJ013041); morphotype D6 was identified as *Trypauchen vagina* (99% sequence similarity to accession number KJ865406); morphotype E3 was identified as *Batis batis* since this morphotype has sequence homology up to 99% of that species (accession number JX193741); morphotype F1-B was identified as *Hippichthys cyanospilos* with sequence similarity of 99% (accession number KU692547.1); and Morphotype G1 was identified as *Hypoatherina valenciennii* with the sequence homology of 99% (accession number AB849027). We were convinced to identify all the above morphotypes into species level because they have high sequences homology to the COI sequences of certain species available on the data bases. Sequences similarity of 98% is reliable enough to be used as a basis for larvae identification into species level because species is a dynamic entity which will show genetic divergence or diversity and sequences divergence of 2% was still low to be considered as basis to place sequence of two individuals into a single species. Our decision to used 98% similarity as species identity was based on the previous study which proved that within-species sequence similarity might range between 97% and 99%, mean that within-species sequence divergence might reach 1-3% (Pegg et al., 2006)

The A1 and D2 morphotypes can only be identified into generic level because they have rather low sequences similarities (94% to *Stolephorus commersonii* (accession number KM236094.1)). This low similarity could be due to that there are not sequenes available in GeneBank nucleotide database for our species. In this case, we did not convenient to place the A1 and D2 morphotypes into *S.commersonii*, although geographical distribution indicates that this species can be found in Indonesia (Froese and Pauly, 2016). Here, we can only put morphotypes A1 and D2 into genus *Stolephorus*. This is because the moderate standard for genetic divergence among species was 3% (Peg et al., 2006). Therefore, it is more save to refer A1 and D2 as *Stolephorus* sp. The Different placement of two individuals larvae (A1 and D2) from the same genetic species into two different morphotypes is possible because each life stage of a single species might show different morphology. It has been noted by Ko et al., (2013) that each life stages showed valuable morphological divergence, this might lead to the placement of each life stages as different species. In one hand, this condition might cause a problem on morphological character-based larvae identification. On the other hand, this difficulty is an important reason to use the molecular marker for proper species level identification of fish larvae.

The E4 morphotype showed sequence similarity as much as 92% to *Zenarchopterus philippinus* (accession number HQ682731.1) and 90% to *Zenarchopterus dispar* (accession number KP194857.1). Therefore, morphotype E4 could only be placed in the genus *Zenarchopterus* and referred as *Zenarchopterus* sp. The placement of morphotypes E4 and also A1 and D1 into generic level were because they have high genetic divergence with COI gene of specific species listed in the NCBI nucleotide database. The morphotype E4 showed sequence divergence as much as 10% to Z. dispar, while A1 and D1 have sequence diverges as high as 6% with *S.commersonii*. We did not sure that those morphotypes could be identified into species level although Nuryanto et al. (2007) has re-
ported that sequence divergences among species from the same genus might exceed 6% but other literature preferred to used level of sequence divergence of 2% (Barber et al., 2002) and 3% (Pegg et al., 2006) as a basis to discriminate among species.

The taxonomic tree was reconstructed using NJ-K2P and MP-cladistic. The two methods resulted in a similar tree topology with similar bootstrap values (Figure 2). It can be seen from the tree that partial sequences of the COI gene can clearly discriminate among morphotypes collected from East Plawangan of Segara Anakan Cilacap Central Java and clearly discriminate among species. Our result proves that partial sequences of COI gene is a reliable molecular marker for fish larvae identification. High resolution of the partial sequences of the COI gene on separating among morphotypes could be due to that this gene has a high mutation rate, which leads to high phylogenetic resolution. A similar result was also reported from various animals such as on Calanoid Arthropod (Hebert et al., 2003b, Australian fishes (Ward et al., 2005; Pegg et al., 2006); Indian fish (Thirumaraiselvi et al., 2015) and grouper species (Nuryanto and Pramono, 2015). The success of our study on larval identification directly provides information on fish species that utilize Segara Anakan as nursery and spawning ground which was formerly not available in that area.

The result of taxonomic tree analysis was supported by K2P genetic distance calculation which showed high genetic distances among morphotypes except for A1 and D1. Complete K2P genetic distances among morphotypes are summarized in Table 1.

It can be seen from Table 1 that pairwise comparison between morphotypes showed high genetic distances with the values range from 19.6% to 27.6%. This high difference among sequences from different morphotypes proved that the COI gene has high resolving power on species discrimination and therefore could create clear topology or branching pattern on taxonomic tree (Figure 2). Our result similar to what was reported by Kurniawaty et al. (2006) on three species of Phlaeothripidae which showed high sequences.

**Figure 2.** Taxonomic tree illustrating morphotype discrimination into species level
Remarks: number above the lines are NJ bootstrap values, number below the lines are MP bootstrap values, 1000 bootstrap replicates)
CONCLUSION

Based on homology test, genetic distances, and taxonomic tree reconstruction, the fragment of cytochrome $c$ oxidase 1 gene showed high accuracy for species discrimination among larvae morphotypes collected in East Plawangan of Segara Anakan Lagoon. Therefore, it can be used as the molecular barcode to verify the validity of morphological identification. East Plawangan is an important aquatic ecosystem as the spawning and nursery ground of various fish species. In order to provide general picture of Segara Anakan as an important ecosystem for fish to spawn and nurse their larvae, it is necessary to do a further research to sample fish larvae from whole part of Segara Anakan.

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