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 Lagon 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 microhabitat 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, larvae 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 larvae (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. Hebbert 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 Esat 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 CAA 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-

tal volume of 50 µl containing 29,8 µl DNA- and RNA-free water; 1X PCR buffer, 0.01 mM of each primer, 0.05 mM of dNTPs, 1 U Taq polymerase, and 4 µl DNA template. Thermal cycles were set as follow: pre-denaturation at 95°C for five minutes, and followed by 35 cycles consisted of one minute denaturation at 94°C, *annealing* at the temperature of 54°C for one minute, and 1.5 minutes extension at 72°C. Final extension was performed at 72 °C for 5 minutes (Ward et al., 2005). Amplicons were migrated on 1% agarose gel and visualized over UV-transilluminator.

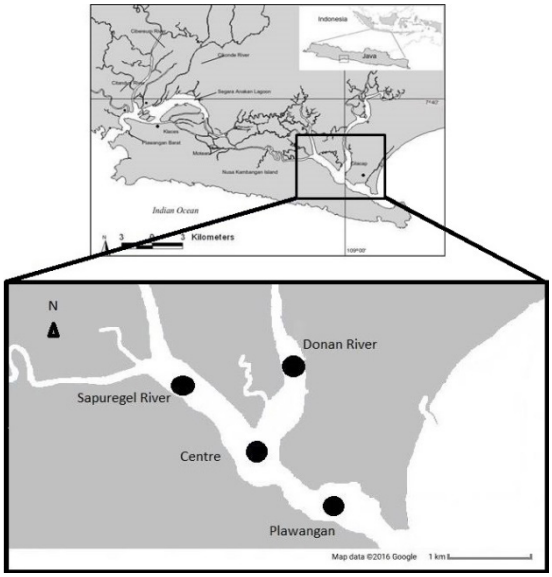

Figure 1. Sampling sites of larvae and water quality parameters (Source: Ardli, 2007 and modified from Google Map)
Remark:
● = sampling site

Sequencing

Clear and strong amplicons were shipped to 1st BASE (www.base.asia.com) for sequencing.

Sequences editing and data analysis

Sequences alignment was performed with the help of Bioedit 7.0.4.1 (Hall, 1999) software and edited manually. Multiple sequences alignments was conducted using ClustalW (Thompson et al.,1994) as implemented in Bioedit. Taxonomic status of the sequences was done through sequences comparison with sequences available in GenBank (NCBI) using *Basic Local Alignment Search Tool* (BLAST) and taxonomic tree reconstruction. The taxonomic tree was reconstructed based on K2P *neighbor-joining* (NJ) and *maximum parsimony* (MP) using the cladistic algorithm with the help of MEGA version 6.0 (Kumar et al., 2008). *Cromileptes altivelis* (Susanto et al., 2011) was used as outgroup comparison. Branching pattern was supported by 1000 *non-parametric bootstraps* replicates.

RESULT AND DISCUSSION

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 diveristy of fish, indicating the important of East Plawangan as the spawning and nursery ground. According to NCDENR (2006) ecosystem inhabitated 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 indviduals 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 upperstream of a river or in the deep sea during spawn and the larvae will move to East Plawangan in order to complete their life cycles. Therefore, precise infromation about the species that use East Plawangan as their spawning and nursery ground can only be obtain through larval identification. However, it is veri dificult to identify fish larvae moprhologically 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 singgle 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 singgle species but at different life stages can be identified as two different species becuase they have different morphology. Therefore, it was reasonable if morphologically we identified as A1 and D2 morphotypes but both morphotypes are belong to a singgle 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 addopted 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 *Butis butis* 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 valenciennei* 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 squences 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 lavae (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

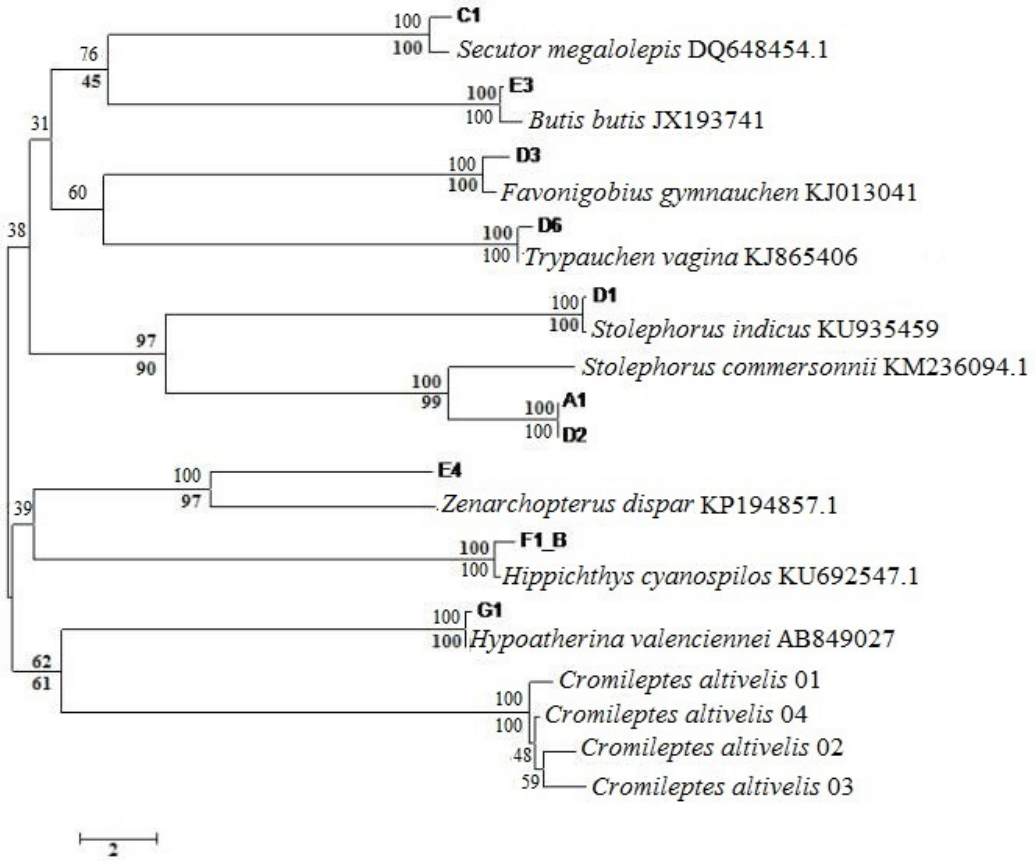

Figure 2. Taxonomic tree illustrating morphotype discrimination into species level
Remarks: number above the lines are NJ bootstrap values, number below the lines are MP bootstrap values, 1000 bootstrap replicates)

Table 1. Genetic distances among morphotypes based on Kimura 2 Parameter model)

Species	A1	C1	D1	D2	D3	D6	E3	E4	F1-B
C1	0.234								
D1	0.211	0.223							
D2	0.002	0.232	0.209						
D3	0.276	0.229	0.276	0.274					
D6	0.251	0.228	0.240	0.249	0.205				
E3	0.253	0.196	0.249	0.251	0.222	0.228			
E4	0.259	0.206	0.262	0.257	0.211	0.232	0.238		
F1-B	0.258	0.259	0.268	0.256	0.250	0.262	0.249	0.236	
G1	0.235	0.232	0.263	0.233	0.247	0.256	0.249	0.245	0.246

divergence among species, Thirumaraiselvi et al. (2015) on fish larvae from Vellar Estuary Tamilnadu India, Pegg et al. (2006) on coral reef fish from Great Barrier Reef Australia, and Ward et al. (2008a) on Australian Chondrichthyans, Ward et al. (2008b) on Asian seabass and Nuryanto et al. (2007) on giant clam. These similarities prove that the COI gene is a reliable marker for species discrimination in the wide range of phyla. There are no certain values of genetic divergences that can be used as the standard to define species status. It seems that different group of animal show the different level of genetic difference. Barber et al. (2002) noted that genetic divergence of 2% is enough to be used for species discrimination Crustacea. Moreover, Nuryanto et al. (2007) reported that variable genetic divergence values were observed among giant clams, with range values from 5 % up to 19%. Thirumaraiselvi et al. (2015) reported that genetic divergence of more than 25% was observed among fish larvae from Vellar Estuary, India.

In the exception to another pairwise comparison, A1 and D2 morphotypes comparison showed the small genetic distance of 0.2%. This means that morphotype A1 and D1 belong to a single genetic species. Pegg et al. (2006) have noted that genetic difference of 1-3% can be found among individuals from single species. Our result validates that morphological identification of A1 and D2 was incorrect. This miss-identification could be due that A1 and D2 belong to different life stages of single species. According to Leis and Carson-Ewart (2004), fish larvae has unstable morphological characters, each life stage has their own morphology that could be significantly different among stages and might lead to miss-identification.

During the filed trips, out of 24 fish larvae morphotypes, it was also observed that the larvae of shrimps, crabs, and jelly fish utilize East Plawangan of Segara Anakan as their spawning and nursery ground. Moreover, it has been reported by Romimohtarto et al. (1991) and Ardli (2007) that Segara Anakan as a whole is an improtant feeding ground for various aquatic biota and specifically noted by White et al. (1989) that at least 45 fish species inhabit Segara Anakan, including East Plawangan. This data provide information that East Plawangan is an important ecosystem for various species to live, feed, spawn, and nurse their larvae. Our result improved the data about the vital of East Plawangan as an ecosystem. Therefore, it is important to conserve East Plawangan in part and Segara Anakan in general for it sustainable uses, either as ecologically or economically important ecosystem.

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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