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Genetic Homogenity of Commerson's Anchovy (*Stolephorus commersonnii*) in Segara Anakan Cilacap Central Java Inferred from PCR-RFLP Markers

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ABSTRACT

Commerson's anchovy (*Stolephorus commersonnii*) is a small pelagic fish that live in a group and its existence is very abundant in Segara Anakan Cilacap. This anchovy is widely consumed by communities live around Segara Anakan. This leads to a high exploitation rate. Exploited populations generally have low genetic diversity. This study aims to evaluate genetic diversity of commerson's anchovy population in Segara Anakan Cilacap inferred from PCR-RFLP of the cytochrome c oxidase 1 (CO1) gene. This study was conducted from January to April 2018 and used survey method by applying random sampling. As many as 30 samples of anchovy were taken. Genomic mtDNA was isolated using modified Chelex method. Partial sequences of the COI gene were amplified using a pair forward commercially available primer. The lengths of 650 base pair of the PCR products were digested with four restriction enzymes. The *Hind*III enzyme produces PCR-RFLP fragment with the size of 416 bp and 234 bp lengths, *Vsp*I produces 435 bp and 214 bp, CO1-TaqI produces 556 bp and 94 bp and *Rsa*I produces 319 bp, 183 bp, and 148 bp fragments, respectively. The PCR-RFLP fragments were obtained from all samples but they produced uniform band pattern for all 30 anchovy individuals. These results indicated that the anchovy population in Segara Anakan Cilacap has monomorphic allele for all PCR-RFLP markers. Hence, it can be concluded that genetic homogenity was observed on anchovy population in Segara Anakan Cilacap as inferred from PCR-RFLP COI gene.

Keywords: allele; anchovy; homogenity; Segara Anakan

INTRODUCTION

Commerson's anchovy (*Stolephorus commersonnii*), which is locally known as "ikan teri", is a popular small pelagic fish in Indonesia. It is widely distributed fish group and can be found in Segara Anakan Cilacap Central Java. This species is commercially sold as salted fish and is highly fished. It has been reported that commerson's anchovy was highly exploited in Belawan waters North Sumatera (Yuanda *et al*., 2017).

Up to present time, there is no study has been done on exploitation rate and genetic diversity of commerson's anchovy from Segara Anakan. In fact, this species is continuously harvested by fishermen of around Segara Anakan. It assumed that commerson's anchovy population undergoes high exploitation which leads over exploitation. It is worried that natural population of commerson's anchovy in Segara Anakan has been depleted and its sustainability can be disrupted in the near future. Declining population can be estimated through population genetic diversity analysis. Depleted population size due to overexploitation might lose their genetic diversity. In contrast, unexploited population tends to have high genetic diversity within population (Frankham *et al*., 2007). It has been proved that overexploitation caused loss of genetic diversity in natural population (Hauser *et al*., 2002; Wirdateti *et al*., 2015). Therefore, it is important to study genetic diversity on commerson's anchovy population in Segara Anakan Cilacap as a tool for estimating exploitation rates of that fish resource.

Genetic diversity within population can be assessed through molecular characterization using DNA-based marker. C*ytochrome c oxidase* I (CO1) is a commonly used genetic marker on population study of animals (Hebert *et al*., 2003). This is due to that the COI gene has high evolution rates (Kurniasari *et al*., 2014). High evolution rate of the COI gene is

believed as reliable marker to differentiate either among populations or among individuals within a population (Nuryanto & Solihin, 2006).

There are various DNA-based markers. One of which is PCR-RFLP (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism*). This marker has been successfully used to show genetic diversity within population, such as in *Polymesoda erosa* from Segara Anakan (Nuryanto & Sastranegara, 2013) and genetic different among giant gourami populations (Nuryanto *et al*., 2017). High genetic diversity on the PCR-RFLP COI gene was observed in oyster *Striostrea mytiloides* populations (Klinbunga *et al*., 2005). Similar result was also reported on shrimp *Astacus leptodactylus* populations (Khoshkholgh & Nazari, 2015). Therefore, it is assumed that PCR-RFLP marker of the COI gene is reliable marker to be used on examining genetic diversity in commerson's anchovy population in Segara Anakan.

Here we developed PCR-RFLP marker of the cytochrome c oxidase 1 gene in order to evaluate genetic diversity within commerson's anchovy population in Segara Anakan, Cilacap, Central Java. The result of this study is expected to be used on estimating of exploitation rate of commerson's anchovy population in Segara Anakan. So, the data can be used as vital information on formulating policies for sustainable use of commerson's anchovy resources in Segara Anakan Cilacap.

MATERIALS AND METHODS

Fish samples were collected during the field trips in April 2016. Samplings were done following the sampling techniques as explained by Nuryanto *et al*. (2017). The obtained fish samples were preserved in 96% technical ethanol (Bratachem). As much as 30 individuals of commerson's anchovy samples were then examined for genetic diversity analysis.

Genomic DNA of the commerson's anchovy was isolated using modified Chelec® 100 method following Kochzius & Nuryanto (2008). Extracted DNAs were migrated in 1% agarose gel. Successful extraction was proved by the appearance of DNA smear on the gel after visualized over ultra-violet light (UV-light).

The fragments of the COI genes were amplified in total volume of 50 µl mixtures. The solutions consisted of 37.4 µl ddH_2O; 5 µl of 1X *reaction buffer* (10x) , 0.02 mM $MgCl_2$, 0.4 mM of dNTPs NZY-mix, 0.4 picomol of each primer and 0.02 Units of NZYTaq DNA Polymerase (5 U/µl). Amplifications process was performed using a pair of primer as follow. Internal forward primer 5'ATCTTTGGTGCATGAGCAGGAATAGT 3' and FishR2 *reverse* primer 5'ACTTCAGGGTGACCGAAGAATCAGAA 3' (Ward *et al*., 2005).

Thermal conditions were as follows. Pre-denaturation stages were conducted on 95^{o}C for 5 min and followed by 35 cycles consisted of denaturation stages on 95^{o}C for 30 s, annealing on 55^{o}C with the duration of 2 min, and extension on 72^{o}C 1.5 min. The final extensions were conducted on 72^{o}C for 5 min and the PCR reactions were stored inside the thermocycler on 8^{o}C for 5 min. The PCR products were estimated to have a length of ± 650 base pair (bp). The PCR products were then migrated on 1% agarose gel and visualized over UV-light and documented in photograph.

Good PCR products were subjected to initial screening to develop PCR-RFLP markers by digesting the amplicons with eight restriction enzymes; i.e. *Eco*RI, *Hind*III, *Taq*I, *HpyF*31, *Hinf*I, *Pst*I, *Vsp*I, and *Rsa*I. These initials screening were performed to select enzymes capable digest the amplicons. Digestion of PCR products were conducted in a total volume of 31 µl mixtures solution. Digestion method was following the protocol from the company (Thermoscientific). The mixtures consisted of 18µl of ddH_2O, 2µl of 10X reaction buffer, 10µl of PCR products, and 1 µl restriction enzymes (1U). The mixtures were put inside 1.5 ml eppendorf tubes and incubated on 37^{o}C for four hours (except for *Taq*I, it was incubated on 65^{o}C) in thermomixer. Thermomixer was set up in 1000 rpm. The digested amplicons were also migrated on 1% agarose gel and visualized under UV-light. Restriction bands pattern were

documented and referred to PCR-RFLP markers.

The PCR-RFLP marker profiles were analyzed descriptively to obtain information on genetic diversity levels of the COI gene on commerson's anchovy population in Segara Anakan, Cilacap, Central Java.

RESULT AND DISCUSSION

Amplification of the cytochrome c oxidase 1 gene. The fragments of the COI gene were successfully amplified from all samples (30 individuals commerson's anchovy). Amplification process resulted in 650 bp length of the COI fragments (Figure 1).

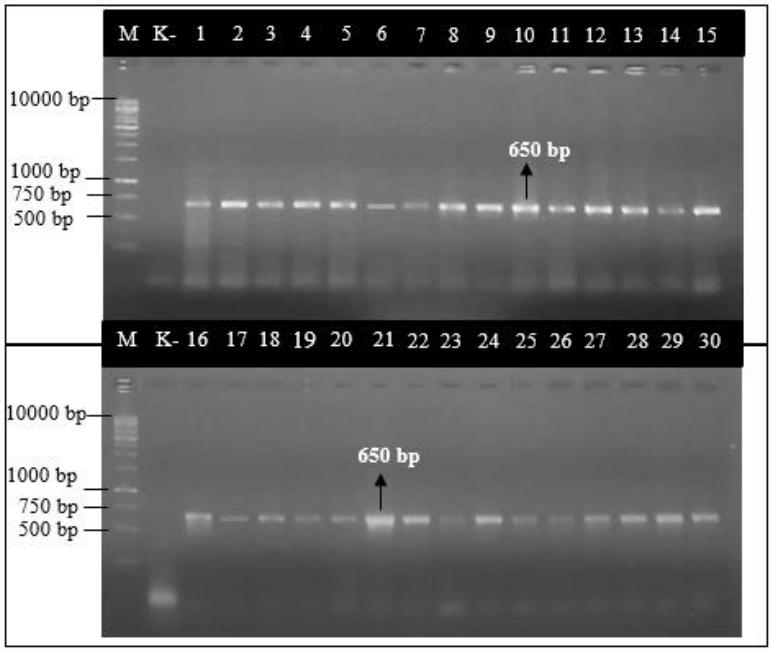

Figure 1. The PCR products of the COI gene from *Stolephorus commersonnii* population

In this study, we obtained a length of 650 bp cytochrome c oxidase 1 fragments. These resulted sizes were a bit longer than the fragment resulted from *Notorhynchus cepedianus* which was only approximately 616 bp length (Ward *et al.*, 2005).

The length differences among the PCR products from different species are common phenomena although these fragments are amplified using a similar primer pair. This is due to that each species has specific sequences in their genome which differ one to another species. This uniqueness leads to different size when it is amplified from different species. Our result showed similar case to several previous studies where different length of the COI gene PCR products were resulted from different fish species (Nuryanto *et al*., 2018; Kusbiyanto *et al*., 2017; Barbuto *et al*., 2010).

We were convincingly assigned that our PCR products were our target fragments of the COI gene rather than pseudo-gene. This belief was based on the result of sequences verification through BLAST method which was performed in previous study by Nuryanto *et al*. (2017).

The resulted amplicons were very specific since band for each individual. This result must be obtained when we used a pair of primer for certain target sequence. Otherwise, the amplification process failed. According to Bahiyah *et al.* (2013) high specificity of successful PCR amplification occurred if single DNA band has resulted.

Detail observation in Figure 1 proved that the intensities of amplicons were different among individuals. These differences could be due to that template DNA for each sample has different quality, especially in DNA amounts. However, we could not explain clearly these DNA amounts since we only performed qualitative measurement of extracted total genome through gel electrophoresis. This result congruences with Rousseau *et al*. (2000) that

the differences in band intensities reflect the quality of template DNA.

Genetic homogenity of *Stolephorus commersonnii* **population.** Genetic homogenity analysis was initialed by digestion of PCR products with restriction enzymes. The initial steps were screening of restriction enzymes which capable to digest the COI gene of *Stolephorus commersonnii*. This screening process involved eight different enzymes.

The screening process found that four out of the eight enzymes were unable to digest the PCR products (*Eco*RI, *Hinf*I, *Hpy*F31, and *Pst*I), while four other enzymes were able to digest the PCR products (*Hind*III, *Vsp*I, *Rsa*I, and *Taq*I). These four restriction enzymes were then used on population genetic analysis of the *S. commersonnii* from Segara Anakan Cilacap, Central Java. The result of initial enzymes screening is presented in Figure 2.

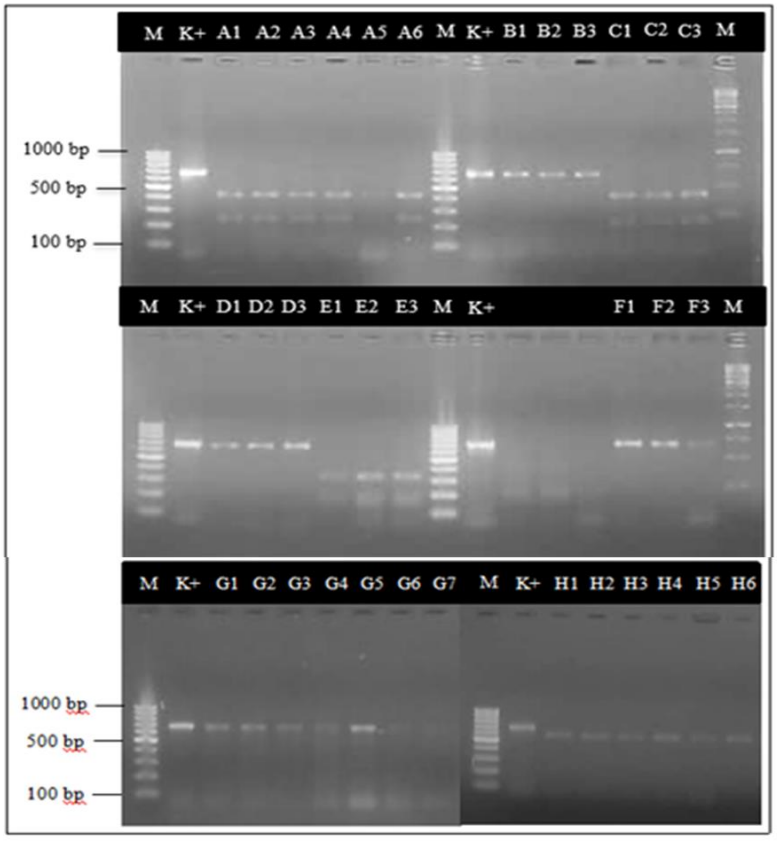

Figure 2. The DNA band pattern after digested by eight different restriction enzymes (M= 100 bp DNA ladder; K+= positive control; A1-A6= digested by *Hind*III; B1-B3= digested by *Eco*RI; C1-C3= digested by *Vsp*I; D1-D3= digested by *Hinf*I; E1-E3= digested by *Rsa*I; F1-F3= digested by *Pst*I; G1-G7= digested by *HpyF*31; H1-H6= digested by *Taq*I)

The results showed that only four digestive enzymes were able to cut the PCR products (Figure 2). This indicates that the COI gene of *S. commersonnii* from Segara Anakan Cilacap, Central Java had restriction sites for those four enzymes. In other words, the four selected enzymes were able to recognize restriction sites in the COI gene of *S. commersonnii*. Whenever recognizable sites are available the enzymes automatically digest the nucleotide sequences if optimal condition is reached and vice versa. According to Nuryanto *et al*. (2017) restriction enzyme only able to recognize a specific sequence. The recognizable site will be digested by the enzyme and produce RFLP markers.

The PCR products digested by *Hind*III enzyme produced two different fragments. These fragments had of 416 bp and 234 bp length. Both fragments were obtained in all individuals and had uniform sizes for all 30 fish samples (Figure 3).

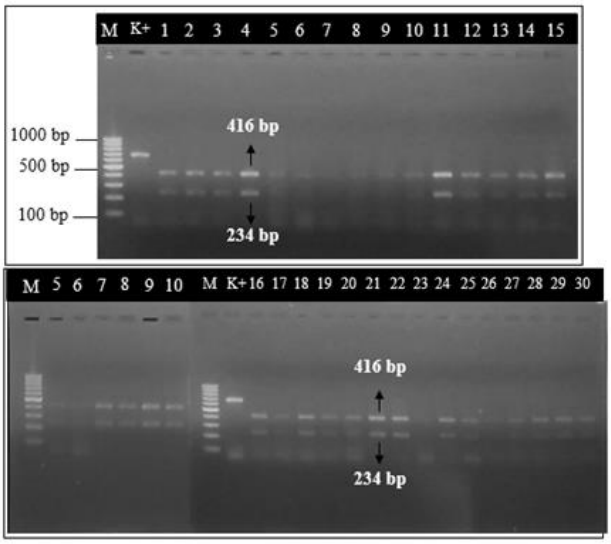

Figure 3. The COI-*Hind*III PCR-RFLP markers of *Stolephorus commersonnii* (M= 100bp DNA ladder; K+= possitive control; 1-30= fish samples)

Two COI-*Hind*III RFLP markers were resulted from *S. commersonnii* population (Figure 3). This indicates that only one restriction site can be recognized by *Hind*III enzyme on the COI gene of that species. Our result is similar to Azizah *et al.* (2015) who obtained two RFLP fragments from giant gourami although with different fragment sizes. Both our result and Azizah *et al*. (2015) results indicated two things. Firstly, the COI gene of fish has single restriction sites since it can only be digested into two fragments. Secondly, that each species has different sequences of the COI gene, although it is amplified using the primer. Therefore, different species will have different length of RFLP fragment after digested by the same restriction enzyme as indicated in *S. commersonnii* and giant gourami (Azizah *et al.*, 2015).

Digestion of PCR products by *Rsa*I indicated that the amplicons were cut into three different PCR-RFLP markers. These markers had length of 319 bp, 183 bp, and 148 bp, respectively. These RFLP markers were also obtained in all samples and had the same sizes for all individuals (Figure 4).

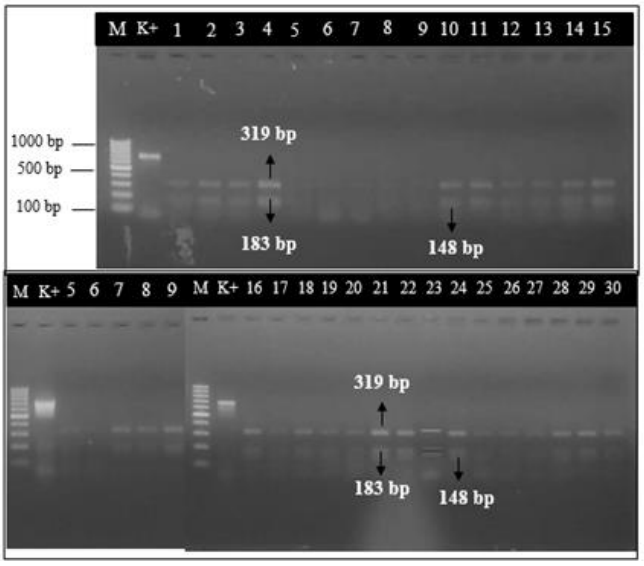

Figure 4. The COI-*Rsa*I RFLP markers of *Stolephorus commersonnii* (M= 100 bp DNA ladder; K+= positive control (PCR product); 1-30= fish samples)

Amplicons digested by *Rsa*I enzyme produced three different COI-*Rsa*I RFLP markers (Figure 4). These markers had sizes of 319 bp, 183 bp, and 148 bp length fragments, respectively. This result indicated that two restriction sites were recognized by *Rsa*I enzyme on the COI gene of *S. commersonnii* population from Segara Anakan. Therefore, it can be digested into three RFLP fragments. The result of three COI-*Rsa*I RFLP markers seems to be rather common on fish samples. This assumption was proposed based on facts that our study and one previous study which also reported two or three COI-RsaI RFLP markers were observed on tuna (Iranawati *et al*., 2016).

The *Vsp*I restriction enzyme was able to digest the COI gene of *S. commersonnii*. The digestion resulted in two different RFLP fragments with the sizes of 435 bp and 214 bp length. These COI-*Vsp*I RFLP markers have also appeared in all 30 samples. The markers had uniform sizes for all individual samples (Figure 5).

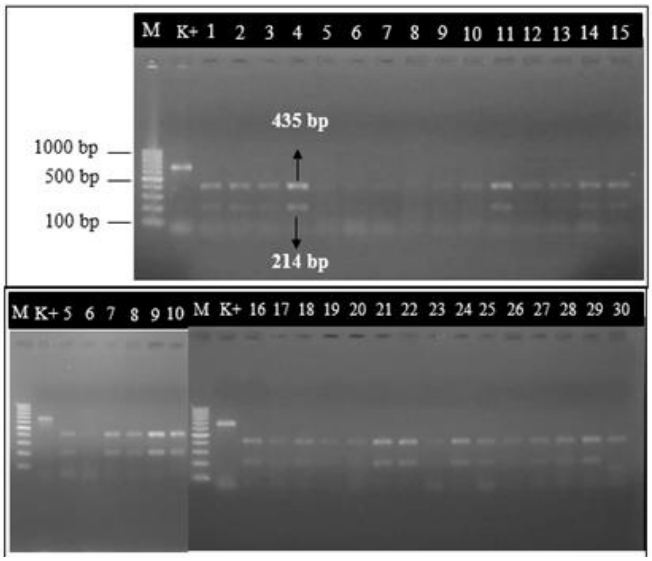

Figure 5. The COI-*Vsp*I RFLP markers of the *Stolephorus commersonnii (*M = 100 bp DNA ladder; K+ = positive control (PCR product); 1-30 = fish samples)

Digestion of 650 bp PCR products of *S. commersonnii* using *Vsp*I enzyme resulted two RFLP markers with the size of 435 bp and 214 bp length, respectively (Figure 5). This result indicated that only one restriction site on the COI gene of *S. commersonnii* was recognized by that enzyme. Our result similar to previous study from Arunprasanna *et al*. (2015) on *Simulium gurneyae* and *Simulium nilgirium* although with different sizes (468 bp and 241 bp). This similarity indicates that the same partial sequences of COI gene in different species has similar restriction site for *Vsp*I although amplified from different species. However, each species has different length of COI fragments and lead different length of RFLP markers after digestion using the same enzyme, as indicated in this study and the study from Arunprasanna *et al*. (2015).

The last RFLP markers were developed by digestion of PCR products using *Taq*I restriction enzyme. The agarose visualization showed that the amplicons of the COI gene from commerson's anchovy can be digested into two different PCR-RFLP fragments, e.g. 556 bp and 94 bp fragments (Figure 6). The both COI-*Taq*I RFLP markers could be obtained from all 30 fish samples and had the same size for all individuals. The 94 bp length fragment could not be seen on the agarose gel. This size was too small to be visualized.

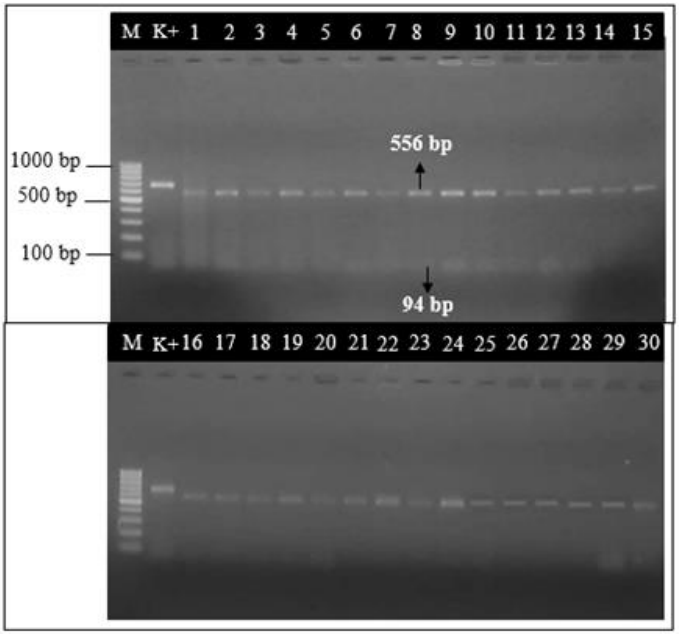

Figure 6. The COI-*Taq*I RFLP markers of the *Stolephorus commersonnii* (M= 100 bp DNA ladder; K+= positive control (PCR product); 1-30= fish samples)

The last RFLP marker resulted from this study was COI-*Taq*I. Two fragments of the COI-*Taq*I RFLP markers were resulted from *S. commersonnii* population in Segara Anakan Cilacap. These COI-*Taq*I RFLP markers had sized of 556 bp and 94 bp length, respectively (Figure 6). This indicated that the COI gene of *S. commersonnii* has only one recognizable site by *Taq*I restriction enzyme.

High different of COI-*Taq*I fragment sizes were also reported on *Nassarius succinctus, N. conoidalis* and *N. sinarus* (Yang *et al*., 2018). He observed COI-*Taq*I fragments of 95 bp length on those three species.

Comparison to other study indicates that our study has different result to what was reported by Domingues *et al*. (2015) on *billfishes*. Domingues et al. (2015) observed two bands on *Kajikia albida*, while three bands were observed on *Tetrapturus georgii, T. pfluegeri, Makaira nigricans, Istiophorus platypterus,* and *Xiphias gladius.*

According to our results, the result from Yang *et al*. (2018) and Domingues *et al*. (2015), it can be assumed that the COI gene has variable number of restriction sites for *Taq*I enzyme over wide range species. Therefore, variable RFLP markers can be observed among species.

Careful and detail observation on Figure 3, 4, 5, and Figure 6 showed that all 30 individuals of commerson's anchovy had similar COI-RFLP markers pattern either for COI-*Hind*III, COI-*Rsa*I, COI-*Vsp*I, or COI-*Taq*I. This indicated that only one allele of all COI-RFLP markers was observed in this study.

It can be argued from the result in Figure 3, 4, 5, and Figure 6 that all the COI-RFLP markers were monomorphics. This argument was based on the fact that the most common allele observed in this study has frequency of 100% while there was not alternative allele or alternative allele was 0%. This also means that no polymorphic locus was observed. According to Nuryanto *et al*. (2017) a locus referred as polymorphic when the most common allele has frequency less than 95%.

The COI fragments in this study did not show any mutation in their restriction sites for the four enzymes used. Therefore, all individuals showed similar PCR-RFLP markers pattern for all enzymes and did not show any polymorphisms. Frankham *et al*. (2002) have noted that polymorphisms might occur if there is alteration in nucleotide sequences due to mutation and lead new allele formed. As a consequence, the population shows genetic diversity.

Monomorphisms in all COI-RFLP markers in this study proved that genetic homogenity was observed in *Stolephorus commersonnii* population in Segara Anakan Cilacap, Central Java. However, this genetic homogenity is applied for all genetic markers outside COI-*Hind*III, COI-*Rsa*I, COI-*Vsp*I, or COI-*Taq*I RFLP markers. In one hand, this genetic homogenity was only indicated the limitation of COI-*Hind*III, COI-*Rsa*I, COI-*Vsp*I, or COI-*Taq*I RFLP markers utilized in population genetic study. This is because RFLP marker can only reflect whether there are variations in restriction sites or not. This marker cannot observe any variation in any sequences outside the restriction sites which can be observed on other genetic markers, such as COI sequences. It was reported by Nuryanto *et al*. (2017) that genetic variation was observed in commerson's anchovy population in Segara Anakan when assessed using COI sequences.

In other hand, genetic homogenity of *S. commersonnii* population in Segara Anakan as indicated either by COI-*Hind*III, COI-*Rsa*I, COI-*Vsp*I, or COI-*Taq*I RFLP markers might reflect the historical demography of the population. It was assumed that our population was historically inherited from a single female remote ancestor. This assumption was come up from the fact that cytochrome c oxidase 1 gene is maternally inherited, haploid, and no recombination events. Therefore, it is a normal phenomenon if single maternally inherited the population showed monomorphism in their mitochondrial DNA (mtDNA). According to Hebert *et al*. (2003) mtDNA is maternally inherited genome. This genome is haploid and no recombination. By these characteristics of mtDNA, the opportunity that the member of population become similar one to the others are high. This phenomenon was observed in our study on *Stolephorus commersonnii* population in Segara Anakan Cilacap.

CONCLUSION

The cytochrome c oxidase 1 gene was successfully amplified from *Stolephorus commersonnii*. Four COI-RFLP markers can be developed from the COI gene of *S. commersonnii* population. However, *S. commersonnii* populations from Segara Anakan Cilacap showed genetic homogenity as assessed by COI-*Hind*III, COI-*Rsa*I, COI-*Vsp*I, and COI-*Taq*I RFLP markers.

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