

Genetic Similarity of Commerson's Anchovy across Segara Anakan Cilacap Assessed Using Randomly Amplified Polymorphic DNA (RAPD) Markers

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ABSTRACT

Segara Anakan areas can be divided into three different regions according to their salinity. Salinity differences suggested that Commerson's anchovy population in that area can be divided into three subpopulations due to genetic differences. Genetic differences among subpopulation can be assessed through a population genetic study using random amplified polymorphic DNA. This study aims to evaluate the genetic variation and differences of Commerson's anchovy (*Stolephorus commersonnii*) collected at three different water salinities in Segara Anakan estuary Cilacap Indonesia. Total genomic DNA was isolated using the Chelex method. Genetic diversity and differences were assessed using RAPD markers and were analyzed statistically using an analysis of molecular variance, as implemented in Arlequin software. The results showed that high genetic diversity was observed within the subpopulations. However, no significant genetic differences were observed among subpopulations which indicate genetic similarity. A high number of offspring are likely to cause high genetic variation within subpopulations. Adult and larvae migration is the cause of genetics similarity across Segara Anakan. Another impressive result is that water salinity did not affect the genetic characteristic of Commerson's anchovy. Genetic similarity of Commerson's anchovy indicates that Segara Anakan forms a single genetic conservation unit.

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Introduction

Commerson's anchovy (*Stolephorus commersonnii*) or locally known as Ikan Teri, is widely distributed fish. It has a geographic distribution from the Indian Ocean to the Fiji Islands.

Commerson's anchovy is a pelagic fish inhabit coastal areas and brackish water. It lives in a massive colony in the water column from 0 meters up to 50 meters depth (Froese & Pauly, 2019). Therefore, it is not surprising that

Commerson's anchovy is abundant in Segara Anakan, Cilacap, Central Java, Indonesia (Nuryanto *et al.*, 2017).

Segara Anakan is an estuary ecosystem directly adjacent to the Indian Ocean. It is a semi-closed aquatic ecosystem separated from the Indian Ocean by the Nusakambangan Island in the western part of Cilacap City. The Segara Anakan has two openings; one in the eastern areas (East Plawangan) and one in the west regions (West Plawangan). Those openings make both Plawangan areas are much affected by seawater input and have high salinity. Water salinity in both locations is ranging from 12 to 28.5 parts per trillion (ppt) (Nugroho *et al.*, 2020; Sastranegara *et al.*, 2020). Conversely, the central areas have salinity ranging from 6 ppt to 25 ppt (Nuryanto *et al.*, 2017). A recent data collected by Sastranegara *et al.* (2020) proved that the lagoon has water salinity below 10 ppt, with the range between 3 ppt to 6.5 ppt. It's mean that lagoon areas have water salinity below the ecological requirement of Commerson's anchovy, that is and salinity conditions ranging from 19.6 to 32 ppt (Froese & Pauly 2019). However, Suprastini *et al.* (2014) and Nuryanto *et al.* (2019a) reported that Commerson's anchovy is also highly abundant in the lagoon areas of Segara Anakan.

Previous studies reported contradictive result about the impact of salinity on the fish genetic constituent. On the one hand, Catanese *et al.* (2016), had found that offshore (low salinity) anchovy population was genetically different to inshore (high salinity) population. Even Whitehead *et al.* (2011) observed significant genetic differences among populations without an obvious salinity barrier. On the other hand, Regmi *et al.* (2016) and Moore *et al.* (2018) noted that there was no significant relationship between salinity gradient and genetic structure of

mosquitofish. Therefore, it is interesting to elucidate which population genetic pattern has occurred on Commerson's anchovy (*Stolephorus commersonnii*) that live in different water salinities in Segara Anakan Cilacap.

Molecular characteristics of a population can be evaluated using RAPD markers (Nuryanto & Susanto, 2010; Sari *et al.*, 2014). Previous studies proved that RAPD is a reliable marker for molecular characterization in fish (Kusmini *et al.*, 2011; Gustiano *et al.*, 2013). A previous study by Muharam *et al.* (2012) observed that two strains of goldfish (*Cyprinus carpio*) have different molecular characteristic based RAPD markers. A survey by Kusmini *et al.* (2016) showed different RAPD profiles among three distinct populations of *Barbonimus schwanenfeldii*. Other studies also reported similar results when applying RAPD markers on the molecular characterization of various species. For example, Kristanto *et al.* (2017) on kissing gourami (*Helostoma temminckii*), Mulyadi *et al.* (2017) on the hybrid of *Osteochilus hasselti* and *Cyprinus carpio*, and Hayuningtyas *et al.* (2016) on three generations of rainbow kurumoi (*Melanotaenia parva*).

An earlier study proved that no genetic differences on the population of Commerson's anchovy from Segara Anakan Cilacap based on PCR RFLP cytochrome c oxidase 1 gene (Nuryanto *et al.*, 2019a) and suggested that *S. commersonnii* formed a single population in Segara Anakan. In contrast, Nuryanto & Sastranegara (2013) reported a different molecular characteristic among *Polymesoda erosa* subpopulation across Segara Anakan with different heavy-metal concentration as revealed by PCR RFLP COI gene markers. Assuming that genetic constituent is strongly affected by the ecological condition and referring to previous studies by Whitehead *et al.*

(2011), Catanese *et al.* (2016), and Moore *et al.* (2018) it seems that different molecular markers, ecological factor, and species might show a different population genetic pattern of organisms. Therefore, we are suggested that RAPD markers and different salinity in the areas of Segara Anakan might show a different pattern of population genetic compared to Nuryanto *et al.* (2019a).

This study aims to evaluate the genetic variation and differences of Commerson's anchovy (*Stolephorus commersonii*) collected at three different water salinities in Segara Anakan estuary Cilacap Indonesia. This research is expected to provide information population genetic pattern of *S. commersonii* in Segara Anakan. The information is essential for fishing management and conservation effort in Segara Anakan Cilacap.

Materials and Methods

Research location and times

Fish samples were collected from three different areas, namely east parts, middle parts, and the lagoon parts of Segara Anakan (Figure 1). A total of 20 fish samples were analyzed from each area. The DNA isolation and amplification were conducted at the Animal Taxonomy Laboratory, Biology faculty, Jenderal Soedirman University. The study was conducted for six months, starting from February 2019 until August 2019.

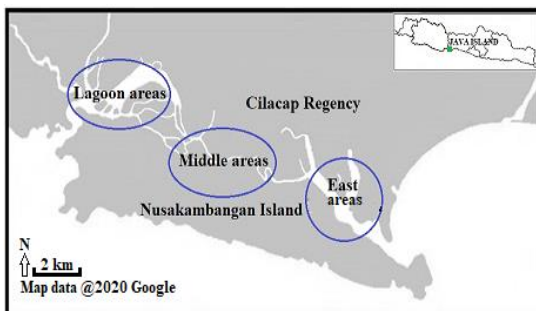


Figure 1. Sampling sites in Segara Anakan

DNA Isolation

DNA isolation was carried out using the Chelex method (Walsh *et al.*, 2013). The reaction was performed in a mixture of 100 μL of Chelex 5%, 5 μL of dithiothreitol 0.1M, and 4 μL of proteinase-K, which was put into a 1.5 ml microtube size. The mixed solution was made for ten samples at a time, so six microtubes of 1.5 mL were used. The mixed solution is then homogenized with a vortex machine for 30 seconds. After being homogeneous, the mixed solution was divided into 60 new 1.5 mL microtubes of 109 μL .

The RAPD markers amplification

Random Amplified Polymorphic DNA (RAPD) markers were amplified with the following procedures. At the preliminary steps, the markers were amplified using 23 of primers. Based on the preliminary results, 4 RAPD primers were selected (Table 1) and were used to analyze the population genetic of *S. commersonii* samples. The primer was selected based on non-contamination and produce clear and strong bands, as shown from preliminary research.

Table 1. A list of selected primers

Name	Nucleotide sequence 5'-3'
OPA 08	GTGACGTAGG
OPA 20	GTTGCGATCC
OPAH 08	TCCCCGTGCC
GEN 14	CGCATTCGCG

The PCR reaction was carried out with a volume of 25 μL . The mixed solutions for one reaction consisted of 17.6 μL ddH₂O, 2.5 μL 10X PCR buffer, 1.25 μL MgCl₂ 50 mM, 1 μL dNTPs, 1.5 μL primer, 0.15 μL Taq polymerase, and 1 μL DNA template. PCR amplification was performed in thermal condition as follow. The pre-denaturation stage was conducted at a temperature of 95°C for 2 minutes and followed by 35 cycles. The cycles were as follow. Denaturation conditions at a temperature of 95°C for 45 seconds, annealing for 45 seconds with the

temperature adjusted based on the melting temperature (T_m) for each used primer (Table 1), elongation at 72°C for 1 minute, then proceed with final elongation at 72°C for 5 minutes.

Electrophoresis and DNA visualization

Electrophoresis was carried out for DNA isolation and PCR amplification products and was done in 1.5% of agarose gel. Electrophoresis was run on 80 volts and 400 mA for 70 minutes. The isolation and PCR products were visualized under a UV transilluminator and documented using 16-megapixel cameras.

Data Analysis

The genetic variations of Commerson's anchovy were estimated based on polymorphism (Θ), gene (h), and locus (π) diversity values. Genetic differences among subpopulations were evaluated using F_{st} and variance component values and compared to their p -value. The polymorphism was analyzed mathematically by dividing an allele by all observed alleles and presented as allele percentage and then compared to the per cent value available in Hartl & Clark (1997). Gene and locus diversities and molecular differences among subpopulations were analyzed statistically. The used statistical tool was an analysis of molecular variance (AMOVA). The analysis was performed in Arlequin ver. 3.5 (Excoffier & Lischer, 2010). Before the variance analysis was conducted, the band pattern data were converted to binary data 0:1 (0= no RAPD bands; 1= RAPD bands are present).

Results and Discussion

Genetic variation

Polymorphism analysis showed that RAPD markers of Commerson's anchovy showed variable values. East subpopulation had a polymorphism

value of 78.21%. Lagoon subpopulation has a value of 66.67%, and middle subpopulation has of 80.77% (Table 2). The obtained values indicate that the used RAPD markers are highly polymorphic for all the three subpopulations. It was because they have the most common allele frequency at a locus in the subpopulation of less than 95%. Hartl & Clark (1997) have stated that a locus is referred to as polymorphic if the most common allele has a frequency below 95%.

High polymorphisms of RAPD markers obtained in the present study are similar to reported results in the previous research in several fish species. For example, high polymorphisms of RAPD markers were observed in goldfish (Muharam *et al.*, 2012), kissing gourami (Kristanto *et al.*, 2017), and rainbow kurumoi (Hayuninftyas *et al.*, 2016). Even, Nuryanto & Susanto (2010) observed high polymorphisms of RAPD markers in *Polymesoda erosa* (Bivalvia) from Segara Anakan Cilacap. Based on the present and those previous studies results, it can be noted that high polymorphisms of RAPD markers are common phenomena in a wide range of animals.

Table 2. Polymorphism and genetic diversity

Areas	Θ (%)	h	π
East	78,21	1 ± 0.02	0.23 ± 0.12
Middle	80,77	1 ± 0.02	0.25 ± 0.13
Lagoon	66,67	1 ± 0.02	0.19 ± 0.10

Note: Θ = Polymorphism

h = Haplotype diversity

π = Locus diversity

It can be seen in Table 2 that the examined subpopulations have a different value of polymorphisms. The phenomenon could be due to that each subpopulation lives in a habitat with different water salinity (East and Middle areas have high salinity, while lagoon areas have low salinity). Our argument was based on the report by Catanese *et*

al. (2016) that water conditions, such as salinity, might become a chemical barrier for the populations.

Table 2 also shown that all three subpopulations have high and similar gene diversity values. The value was of 1 ± 0.0158 . The value indicates that Commerson's anchovy has high genetic diversity within subpopulations because of the value close to 1. According to Nuryanto *et al.* (2019b), gene diversity value of 1 indicates high gene diversity of a population. High gene diversity of the population based on RAPD markers was also reported in giant gourami (Sari *et al.*, 2014) and *Polymesoda erosa* from Segara Anakan (Nuryanto & Susanto, 2010).

Comparison to other studies showed that the present study obtained a higher gene diversity than that of Indian anchovy (*Stolephorus indicus*) studied by Sukumaran *et al.* (2019), and European anchovy (*Engraulis encrasicolus*) (Viñas *et al.*, 2014).

Locus diversity (π) was ranged from 0.19 ± 0.10 in lagoon subpopulation to 0.25 ± 0.13 in the middle subpopulation. The detail values for all subpopulations are presented in Table 2. The values indicate that all *S. commersonnii* subpopulations in the Segara Anakan have a high locus diversity of RAPD markers because of the values higher than 0.1. According to Nuryanto *et al.* (2019b), locus diversity of 0.1 indicated high locus diversity. Loci diversity, as obtained in the present study was higher than Indian anchovy (*S. indicus*), and European anchovy (Viñas *et al.*, 2014; Sukumaran *et al.*, 2019).

Genetic variability that occurs can be caused by mutations, random mating, migration, and recombination (Melian *et al.*, 2012). A population that has a fast reproductive rate and produces a large number of offspring tends to have a high level of genetic variation because it allows random interbreeding.

Interbreeding between populations might increase genetic diversity because of genetic recombination (Melian *et al.*, 2012). Therefore, it is reasonable that the present study observed high genetic diversity because Commerson's anchovy is among fish species with high reproductive rates and produces an enormous number of offspring in one spawning. According to Froese & Pauly (2019), Comerson's anchovy belongs to the Clupeoid group that lives in a large school. These fish groups also have fast reproductive property. Moreover, it can produce a large number of offspring, mature before one year, and spawn throughout the year. It is suggested that all those reproductive properties of Commerson's anchovy have contributed to a high genetic variation in anchovy (*S. commersonnii*) subpopulations from Segara Anakan.

Genetic difference analysis

Pairwise comparison showed no genetic differences among the three anchovy subpopulations in Segara Anakan despite lives in different levels of water salinity. Genetic similarity was indicated by a variance component value of 0.00 and the fixation index (Fst) of 0.00 among subpopulations (p -value 1.000). According to the result presented in Table 3, it is undoubted to state that *S. commersonnii* in the Segara Anakan formed a single genetic conservation unit.

Table 3. Amova analysis

Source of variation	d.f	Sum of squares	Variance components
Among populations	2	1	0.00 Va
Within populations	57	28.50	0.50 Vb
Total	59	29.50	0.50
Fst	0.00		
p-value	1.00		

It can also be seen in Table 3 is that variation has occurred within subpopulations (Vb = 0.500). That data supports the result of gene diversity and

locus diversity analysis that high molecular variation was observed within subpopulations, as explained in the previous section. The phenomena could be because the salinity values within each subpopulation are also high, which lead various alleles to adapt to that condition.

Comparison to previous studies suggested that high genetic diversity within subpopulations is common in various animals and using variable molecular markers (Chandra *et al.*, 2010; Nuryanto & Susanto, 2010, Nuryanto & Sastranegara, 2013; Sari *et al.*, 2014).

This study observed that water salinity does not significantly affect the genetic constituent of Commerson's anchovy in the Segara Anakan. It is proven by no statistical difference in the variance component among subpopulations and extremely low F_{st} values (p-value 0.000, Table 3). This result consistent with and strengthen the previous study using the PCR-RFLP COI marker by Nuryanto *et al.* (2019a) that genetic homogeneity was observed in Commerson's anchovy in Segara Anakan. Similar results were also reported by Damerau *et al.* (2012), that low and even absence of molecular differences among populations are common in marine fish.

This study obtained a different result from Catanese *et al.* (2016), which shows that differences in water salinities could affect the level of genetic diversity. The differences because both studies be explained at least by two arguments. First, the present study used Commerson's anchovy as a research object, while Catanese *et al.* (2016) used European anchovy. Different species will have a different response to environmental conditions. That difference might cause in different genetic response as a result of different natural selection factors. Second, the present study used RAPD as a molecular

marker, whereas Catanese *et al.* (2016) used SNPs markers. The SNPs markers have a high rate of mutation. That condition could be the cause of molecular differences among herring populations, which are collected from different water salinity ecosystems.

The absence of molecular differences among Commerson's anchovy subpopulations in East, central, and the lagoon areas indicates high gene flow. Gene flow can be caused by the euryhaline nature of anchovies that can live in waters with low to high salt levels (Silva *et al.*, 2014a). This tolerant trait makes anchovies have a wide distribution in the waters. Broad distribution is also supported by migration, both in adult fish and their planktonic larvae (Silva *et al.*, 2014b). Migration of adult anchovy often occurs in feeding areas (feeding ground) (Catanese *et al.*, 2016). Migration in adult anchovy also occurs when the fish will spawn. Anchovy will migrate from the estuarine area to the area near the beach to spawn. Planktonic larvae of anchovies will then migrate back to the estuarine site until the juvenile and adult phases (Correa-Herrera *et al.*, 2017). Based on the nature of *S. commersonnii*, the difference in water salinity between East areas and Lagoon areas does not inhibit anchovy migration. Besides, geographic distance east areas and lagoon might also not be a barrier to anchovy distribution. The distribution of planktonic larvae can also be facilitated by tidal waters (Silva *et al.*, 2014a). Eastern areas directly connected to the ocean and high tides could carry anchovy planktonic larvae to the middle and lagoon areas (Nuryanto and Sastranegara, 2013).

No genetic differences among the three subpopulations of Commerson's anchovy populations (*S. commersonnii*) across Segara Anakan indicate that Commerson's anchovy across Segara

Anakan forms a single gene pool. In term of conservation, the result proved that *S. commersonii* constituted a single genetic conservation unit across Segara Anakan and suggested a single policy for Segara Anakan Conservation. However, further research using more sensitive molecular markers, such as microsatellite, is still needed to obtain a comprehensive figure about population genetic of Commerson's anchovy populations in Segara Anakan. It is because complete information will provide a strong scientific basis for the management of Commerson's anchovy fishery in the Segara Anakan or even generally for conservation Segara Anakan as an aquatic ecosystem.

Conclusion

This study concludes that high genetic variations were observed at three subpopulations of anchovy (*S. commersonii*) in Segara Anakan Cilacap. There is no genetic differences among subpopulations indicate that Segara Anakan, Cilacap formed a single genetic conservation unit.

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