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GENETIC VARIABILITY OF *POLYMESODA EROSA* POPULATION IN THE SEGARA ANAKAN CILACAP

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ABSTRACT

Mud clams, *Polymesoda erosa*, in the Segara Anakan Cilacap are highly exploited by the local communities for daily consumption. This is presumed causing population decline and potentially causing loss of genetic diversity. Genetic diversity level within population can be obtained by population genetic study using molecular marker such as randomly amplified polymorphic DNA (RAPD). Here we amplified RAPD marker using ten arbitrary primers to assess genetic diversity of *P. erosa* population in the Segara Anakan Cilacap to provide genetic data for its sustainable use. The results proved that the use of RAPD marker has high polymorphisms. The mud clam population also showed a high level of heterozygosity and genetic diversity. This has important implication for the management plan towards sustainable use of *P. erosa* in the Segara Anakan Cilacap.

Keywords: mud clam, RAPD, polymorphism, genetic diversity.

INTRODUCTION

Mud clams, also known as mangrove clams of the genus *Polymesoda*, are widely distributed in the mangrove habitat. Three species of mangrove clams belonging to the genus *Polymesoda* are reported from the Indo-Pacific regions (Ingole *et al.* 2002). They are *Polymesoda erosa*, *P. bengalensis* and *P. expansa*. *P. erosa* is distributed almost in all Indo-Pacific coastal regions, including in the Segara Anakan Cilacap, Central Java, Indonesia.

Mangrove clams, *P. erosa*, have considerably big size with more flesh body. Adult individuals of *P. erosa* might reach 11 cm in shell size (Gimin *et al.* 2004). Therefore, it is not surprising that these clams are utilized as one of food resources by local community who lives close to the coastal region (Meehan 1982) including the community near the Segara Anakan areas, Cilacap (Dudley *et al.* 2000). Moreover, this mangrove clam has a good quality to be reared in mariculture (Morton 1976).

In the Segara Anakan areas, *P. erosa* are collected by the fishermen to fulfill their daily consumption (livelihood) or even for sale at local market in Cilacap and surrounding areas. This is indicating that mud clams, *P. Erosa*, in the Segara Anakan is

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highly exploited. On the other hand, the size of Segara Anakan areas has been reduced due to land conversion, sedimentation, and so on (Hanley 2000). Formerly, Segara Anakan had around 4603 ha of mangrove areas. However, illegal logging had caused the area of mangrove forest became smaller and smaller; it was around 1454 ha between 1974 and 1987. In 1989, about 16.5 ha of mangrove forest in the Segara Anakan areas had been converted into shrimp ponds. The decrease of mangrove area in the Segara Anakan is also caused by sedimentation which is coming from Citanduy, Cibeureum, and Cikonde Rivers (Sastranegara 2004).

A high rate of exploitation and declining of the Segara Anakan areas are presumed to have direct impact on its animal communities, including *P. erosa* population. The alteration of natural communities could be reflected by the changes of biodiversity and abundances (Kempton 1979), especially on the benthic organisms (Lambshead *et al.* 1983). Biodiversity is divided into three levels: (1) intraspecific diversity, it is well known as genetic diversity within population, (2) species diversity and (3) ecosystem diversity (Fe´ral 2002). Hauser *et al.* (2002) noted that overexploitation may cause a loss or decrease of genetic diversity within population which potentially affects adaptation capability, population resistance and productivity.

Genetic diversity level within population can be obtained by population genetic study using molecular marker such as randomly amplified polymorphic DNA (RAPD). A study on population genetic is intended to evaluate genetic variation of the population under study. Genetic variation level within a population is one of the vital information that supports conservation effort (Lande & Barrowclough 1998, Simberloff 1988, Suryadi 2002). This is due to genetic diversity which is needed by the species to maintain their reproductive capability and adaptation to their environmental alteration (O'Brien 1994, Fe´ral 2002, Reed & Frankham 2003) including their resistance to several types of diseases (Tarpy 2003, Hughes & Stachowicz 2004). In other words, species need to be variable enough in their genetic resources to be adaptive to their environment. Moreover, information on genetic diversity data is very important to support the utilization of nature resources. Indriani *et al.* (2002) noted that higher variation will lead to a higher probability to obtain organism with the expected character. Information about species abundance and diversity are very important for the sustainable use of natural communities in a given ecosystem. According to Hadie *et al* (1998), restoration and conservation effort should consider local populations; firstly, because it is related to local adapted gene, the second consideration is to introduce other genes from metapopulation with better intraspecific genetic variation. This agrees with Savolainen (1994) that genetic variation is an important component of genetic diversity conservation, because it is directly related with adaptation.

A study on genetic diversity can be done using DNA and proteomics. Both markers are relatively stable or not greatly affected by the environment (Purwanto *et al.* 2002). However, DNA marker provides more advantages compared with proteomics markers. The advantage of DNA as genetic marker is that it provides direct information on variation within a given population (Ward & Grew 1994).

Several DNA markers have been developed to study genetic diversity of population. They are DNA sequences (nuclear DNA and mitochondrial DNA),

RFLP, AFLP, RAPD and so on (Fe'ral 2002). Random amplified polymorphic DNA (RAPD) analysis is an analysis using arbitrary oligonucleotide with 10 bases length as a primer to amplify DNA fragment from discrete genome. RAPD analysis provides a cheaper and faster technique compared with restriction fragment-length polymorphism (RFLP) analysis (Penner *et al.* 1993). Moreover, this method also provides an alternative method to identify DNA polymorphisms related to commercial characters.

RAPD technique results to a huge number of DNA fragment the majority of which are individual specific (Welsh & McClelland 1990, Williams *et al.* 1990). If heritability pattern is verified, RAPD patterns can also be used for population genetic analysis (Williams *et al.* 1990) that includes intraspecific genetic variation analysis. However, there is a possibility that dominancy occurred on RAPD marker. If this is the case, genetic variation may be hidden and small fragments are not visualized. In order to avoid this case, during the research primers were used which produced consistent RAPD marker and showed polymorphism on bivalve specimen following Rego *et al.* (2002) and Holmes *et al.* (2004). The potential of RAPD marker used on population genetic analysis had been reported on several organisms (Hadrys *et al.* 1992). This technique has proven the presence of genetic polymorphism on intraspecific level, such as on oyster (Hirschfeld *et al.* 1999, Klinbunga *et al.* 2000, Klinbunga *et al.* 2001), mussels (Rego *et al.* 2002), scallop (Patwary *et al.* 1994), abalone (Huang *et al.* 2000), zebra and quangga mussels (Stepien *et al.* 2002), and bivalve *Abra tenuis* (Holmes *et al.* 2004). Baozhong *et al.* (2006) proved that RAPD marker provide more information about genetic diversity within population compared with enzymes electrophoresis, especially on scallop (*Chlamys farreri)*. Moreover, RAPD marker can also be used in species identification (Rego *et al.* 2002, Marin *et al.* 2007).

RAPD

This study is aimed to obtain information on RAPD marker with consistent and polymorphic band and genetic diversity data on *P. erosa* population in the Segara Anakan Cilacap. The obtained data is valuable to evaluate the genetic potential of *P. erosa* resources in the Segara Anakan Cilacap, so it can be used as a basis to create a strategy of sustainable use of the clam resources.

MATERIALS AND METHODS

Sample collection

Mantle tissues of *P. erosa* were collected from the Segara Anakan Cilacap during the field trips in 2009. DNA analysis was carried out at the Plant Biology Laboratory, Research Centre for Life Sciences and Biotechnology, Bogor Agriculture University. A small piece of mantel tissues were cut off from each specimen with the help of forceps and scissors. Mantle Tissue samples were preserved on 96% of ethanol and stored at 4°C until DNA analysis.

DNA isolation and RAPD Amplification

Total genomic DNA was isolated using Chelex® method from 20 individuals and following the protocols from Walsh *et al.* (1991) with slight modification as follows:

approximately 2x2x0.3 mm^3 muscle tissue was cut off and put in Eppendorf tubes of 1.5 ml. Add 100 µl Chelex 5%, 5 µl Dithiotreitol 100 mM and 4 µl Proteinase-K into the tubes. The samples were incubated for four hours at 55 °C. Afterwards, the samples were centrifuged at 13.000 rpm for 3 minutes. The supernatants were transferred into new tubes and finally incubated at 95°C for 5 minutes. The RAPD markers were amplified using the following ten primers: GEN11 TGCGCGATCG, GEN12 CAGGGTCGAC, GEN13 ACGGTGCCTG, GEN14 CGCATTCCGC, GEN15 GAGATCCGCG, GEN16 GGACTCCACG, GEN17 ATCTCCCGGG, GEN20 CAGACACGGC, and GEN23 GTGTAGGGCG, GEN24 CGGGTCGATC (Aranisi & Okimoto 2004).

PCR reactions were carried out in a total volume of 25 µl containing approximately 0.15 µl DNA Taq polymerase; 2.5 µl PCR *reaction buffer*; 2,5 µl Dithiotreitol (100 mM); 1 µl dNTPs (5 mM); 1 µl each primer (1 mM); 3,5 µl template DNA and ultrapure water up to 25 µl final volumes (modified from Holmes *et al.* 2004).

During preliminary study, thermal cycles were as follows: denaturation step for 3 minutes at 94 °C, and followed by 35 cycles: one minute at 94 °C, 2 minutes at 40 °C for annealing, and one minute at 72 °C for elongation Final extension was carried out at 72 °C for 5 minutes (Rego *et al.* 2002). This protocol was modified to consist of an initial denaturation at 94°C for 3 minutes, followed by 45 cycles of 10s at 94°C, 10 s at 38°C and 90 s at 72°C, and then a final extension at 72°C for 10 minutes for RAPD-PCR analysis using 4 selected primers. A 20µL portion of amplicon was migrated at 100 Voltage on a 1.0% agarose gel and visualized under UV illumination Gel Documentation.

Data Analysis

Genetic diversity level were analyzed using the following formula: averages polymorphic loci (*P*) = number of polymorphic loci divided by total loci time hundred percent. A locus said to be as polymorphic loci if the frequency of the most common allele is less than 0.95 (Hartl & Clark 1997). $H=\Sigma\ (1\Sigma\chi_i^2)/n$, χ_i is i_{th} allele frequency at χ locus within a given population; *n* is total number of loci under study (Baozhong *et al.* 2006). Gene diversity (H) and it standard deviations are estimated to evaluate genetic diversity of *P. erosa* population. The calculation was performed with the help of Arlequin software (ver. 2.0; Schneider *et al.* 2000) following the recommendation from Nei (1987). Before the data were applied to the software, the RAPD's band patterns were changed into binary data (0:1; 0 mean the fragments were not present and 1 mean present).

RESULTS AND DISCUSSIONS

During pre-experiment periods, we had also designed special program to amplify DNA to obtain high genetic diversity on PCR-RAPD marker analysis. Optimization of thermal cycle condition was done to obtain reproducible fingerprint. Optimization was conducted as follows: pre-denaturation for 2-3 minutes, followed by 35 cycles: one

minute denaturation at 94°C, *annealing* for 10 - 30 seconds at 38-40 °C, elongation at 72°C for one minute and final extension for 5 minutes at 72°C. Finally, thermal cycle was performed as follows: pre-denaturation at 94 °C for 3 minutes and followed by 45 cycles as follows: denaturation steps at 94 °C for 10 seconds, *annealing* steps for 10 seconds at temperature of 38 °C, and extension steps for 1.5 minutes at 72°C, further extension was performed for 10 minutes at 72 °C. However, during those periods, RAPD marker could only be amplified from seven individuals out of twenty isolated individuals. Therefore, the analysis was then continued based on seven individuals of mud clam.

In the beginning of the study, we used ten arbitrary primers to amplify RAPD marker from *P. erosa* tissue samples. Four out of the ten arbitrary primers could amplify RAPD marker successfully. The successful primers were GEN13, GEN15, GEN23, and GEN24. However, only three out of four primers resulted to RAPD markers with clear and clean band patterns. They are GEN15, GEN23, and GEN24. GEN13 primer resulted unclear band pattern of RAPD markers. On a same reaction condition, the main bands of RAPD product using GEN15, GEN23, and GEN24 primers has shown a similar and consistent band patterns after several PCRs and electrophoresis. Therefore, GEN15, GEN23, and GEN24 primers can be further used for RAPD analysis on *P. erosa* tissue samples.

A total of 23 RAPD GEN24 using selected primers (GEN15, GEN23 and GEN24) with the size ranges from 250 to 1500 base pairs (bp). However, among seven individual samples, GEN15 primer only amplified RAPD markers from 5 individuals. Using this primer, RAPD marker only appeared from individual number 2, 3, 4, 6, and 7, while on individual number 1 and 5 did not (Table 1). GEN 23 and Gen24 primer were able to amplify RAPD markers from all analyzed individuals.

Table 1. The selected primers for genetic diversity analysis of *P. erosa* population from Segara Anakan Cilacap.

Primer	Number of Amplified Band	Number of Band per Individual	Number of Monomorphic Band	Number of Polymorphic Band	Polymorphism %
GEN15	6	0 - 4	0	6	100
GEN23	7	2 - 4	0	7	100
GEN24	10	3 - 4	0	10	100
Total	23		0	23	100

Amplification using GEN15 primer resulted to six RAPD markers with the size range of 300 to 1250 bp. GEN23 primer was able to amplify seven RAPD markers with the size between 250 and 750 bp. Whereas amplification using GEN24 primer resulted to ten RAPD markers with the size range of 350 to 1500 base pairs. The phenotype distribution of RAPD markers on *Polymesoda erosa* population from the Segara Anakan Cilacap are summarized on Table 2.

Table 2. Phenotype distribution based on RAPD markers frequency on *Polymesoda erosa* population from the Segara Anakan, Cilacap

Marker	Number of Phenotype for each marker	Distribution
GEN13-1250	2	0.091
GEN13-1000	2	0.091
GEN13-600	2	0.091
GEN13-500	1	0.045
GEN13-400	3	0.136
GEN13-300	4	0.182
GEN23-750	2	0.091
GEN23-700	1	0.045
GEN23-600	3	0.136
GEN23-500	3	0.136
GEN23-400	5	0.227
GEN23-300	5	0.227
GEN23-250	5	0.227
GEN24-1500	1	0.045
GEN24-1300	1	0.045
GEN24-1250	5	0.227
GEN24-1100	2	0.091
GEN24-800	3	0.136
GEN24-750	2	0.091
GEN24-700	1	0.045
GEN24-625	6	0.273
GEN24-450	2	0.091
GEN24-350	1	0.045
Total	23	

Genetic diversity analysis of all the 23 RAPD markers showed very high polymorphisms (100 %). On gene level, according to the result of genetic diversity analysis using Arlequin software, *Polymesoda* population from the Segara Anakan showed high genetic diversity (1.000 ± 0.076) (Table 3). High gene diversity also proved that the used RAPD markers showed high heterozygosity. Lynch and Miligan (1994) noted that at Hardy-Weinberg equilibrium gene diversity is equivalent with expected heterozygosity. Therefore, mud clams *P. erosa* from the Segara Anakan areas has high genetic diversity and heterozygosity.

Table 3. Analysis of standard genetic diversity using Arlequin Software

No. of gene copies	Number of haplotypes	Number of loci	Number of usable loci	Number of polymorphic sites	Gene diversity
7	7	23	23	23	1.0000 ± 0.0764

From culture and conservation point of view, high genetic diversity, polymorphisms and heterozygosity of *P. erosa* population are very advantageous. It is due to genetic diversity that can be used as an indicator of adaptive potential related to environmental alteration (O'Brien 1994). Therefore, it is expected that high genetic

diversity, polymorphisms and heterozygosity of *P. erosa* population from the Segara Anakan could indicate high adaptation of those population to their environment. A high genetic diversity might improve population resistance (Hughes & Stachowicz 2004; Tarpy 2003). In contrast, loss of genetic diversity has deleterious effect on population fitness (Reed & Frankham 2003).

Based on its high genetic diversity, at the present time, we could say that the population of *P. erosa* in Segara Anakan Cilacap is still in a save condition. This high heterozygosity, polymorphism, and genetic diversity might indicate normal exploitation. However, we have still to pay attention to exploitation rate of *P. erosa* population in the Segara Anakan Cilacap to avoid a worse effect of ove-rexploitation in the future, since this clam is exploited daily and continuously by the fisherman. Daily and continuous exploitation will lead to over-exploitation. Over-exploitation might lead to a loss of genetic diversity on those populations. This condition has a further consequence on sustainable use of those clams in the Segara Anakan Cilacap. Ledig (1992) and Hauser *et al.* (2002) noted that over-exploitation has caused a deep loss of genetic diversity within population. This situation has adverse effect on the sustainability of population because loss of genetic diversity has deleterious effect on population fitness (Reed & Frankham 2003). Conversely, a low genetic diversity might lead to low potential adaptation, population resistance and low reproductive capacity (Hauser *et al.* 2002), whereas high genetic diversity might improve population resistance (Hughes & Stachowicz 2004; Tarpy 2003).

CONCLUSIONS

According to our result and discussion it can be concluded that: 1) three out of four selected primers were able to amplify RAPD marker and resulted to polymorphic bands; 2) *Polymesoda erosa* population from the Segara Anakan Cilacap has high heterozygosity and genetic diversity; 3) the exploitation of *P. erosa* in Segara Anakan is still at normal rate.

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