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Bionatura-Jurnal Ilmu-ilmu Hayati dan Fisik
ISSN 1411 - 0903
Vol. 15, No. 3, November 2013: 184 - 189

MOLECULAR CHARACTERISATION of *Polymesoda erosa* (Solander, 1786) INHABIT TWO DIFFERENT HABITATS

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ABSTRAK

Respon awal dari organisme terhadap polusi adalah detoksifikasi melalui proses fisiologi, namun sebenarnya, respon sudah terjadi pada level molekuler. Pada level molekuler, bahan pencemar dapat menyebabkan kerusakan kromosom atau mutasi DNA. yang dapat mengakibatkan hilang atau munculnya situs restriksi. Oleh karena itu, sangat mungkin utnuk melakukan deteksi pencemaran menggunakan penanda molekuler seperti PCR-RFLP gen sitokrom c oksidase 1. Penelitian ini dilakukan menggunakan metode survey dengan pengambilan sampel secara *purposive random sampling*. Sampel kerang totok dikoleksi selama sampling pada bulan April 2011 di Sungai Donan dan Segara Anakan Cilacap. Enzim restriksi yang digunakan sebanyak 10 buah. Enzim terpilih, kejadi-an mutasi, dan karakter molekuler analisis secara deskriptif berdasarkan kemunculan dari fragmen RFLP pada gel agarosa. Hasil penelitian menunjukan bahwa enzim yang dapat memotong gen sitokrom c oksidase 1 pada Polymesoda erosa dari Segara Anakan Cilacap dan menghasilkan fragmen RFLP adalah *Alu1* dan *Vsp1*, pemcemaran logam berat khususnya Pb pada sedimen dapat mengakibatkan mutasi basa nukleotida pada gen sitokrom c oksidase 1 dari Polymesoda erosa, ini gen sitokrom c oksidase 1 dapat digunakan sebagai penciri molekuler pada populasi *Polymesoda erosa* yang hidup diperairan tercemar dan tidak tercemar, dan populasi kerang totok dari segara anakan cilacap memiliki keragaman genetic yang tinggi meskipun jika dilihat dari masing-masing subpopulasi ada subpopulasi yang tidak beragam dan ada yang beragam.

Kata Kunci: Polusi, *Polymesoda erosa*, sitokrom c oksidase 1, PCR RFLP, enzim restriksi

ABSTRACT

The first organismic response on pollution is detoxification through physiological processes. On molecular level, pollutant causes chromosome damage and DNA mutation. DNA mutation might lead to a loss or form of restriction sites. Therefore, it is possible to detect pollution using PCR-RFLP marker such as using cytochrome c oxidase I gene. This study used survey method by applying purposive random sampling. The samples were collected during the field trip in April 2011 at Donan River and Segara Anakan Cilacap. Ten restriction enzymes were used. Selected enzymes, mutation event, and molecular characters were defined descriptively based on the appearance of restriction fragment on agarose gel. Genetic diversity h was estimated with the help of Arlequin software version 2.0. The result showed that *Alu1* and *Vsp1* could cut the PCR product, heavy plumbum (Pb) pollution on sediment caused nucleotide mutation on cytocrhome c oxidase 1 gene, cytochrome c oxidase gene 1 can be used as molecular character to differentiate *Polymesoda erosa* inhabit heavily and less plumbum polluted ecosystem, and generally *Polymesoda erosa* population at Segara Anakan showed high genetic diversity. However, if we come into detail on each sampling site, some showed low genetic diversity while other sites showed high genetic diversity.

Key words: Pollution, *Polymesoda erosa,* cytochrome c oxidase 1, PCR RFLP, restriction enzyme

INTRODUCTION

Segara Anakan Cilacap is a lagoon ecosystem bordered by mangrove forest. This lagoon located in western part of Cilacap, north of Nusakambangan Island south coast of Central Java. Segara Anakan is an estuary ecosystem which accept input from several rivers in Central and West Java, such as Citanduy, Cibeurem, Cikujang, Cikonde, Kayu Mati, Ujung Alang, Dangkal, Kembang Kuning, Sapuregel, and Donan. Segara anakan areas has geographic coordinate of 07º34'29.42"-07º47'32.39" ana 108º46'30.12"-109º03'21.02" (Ardli and Wolff, 2008).

In one hand, Donan River located at geographic coordinate of 108°01'45"-109°59'18" dan 07°38'45"-07°43'56". This river located closed to several industries in Cilacap, e.g. PT Holchim Indonesia Tbk, PT UP IV Pertamina, Cilacap Industrial areas and Tanjung Intan Port (White et al., 1989). Donan River is also used for international transportation for industrial raw material and product. It is therefore, both Segara Anakan and Donan Rivers is expected represent polluted and unpolluted habitat.

Two kinds of pollutant may enter an ecosystem, that is organic and unorganic material, including heavy metal. Small amount of pollutant play important role on physiological processes of organism. The problem come if a high number of pollutant exceed the capability of the orgsnisms to neutralize them. This may lead to cellular damage or even followed by individual mortality (Avery, 2001; Gaetke and Chow, 2003).

Organimal response on pollutant ranges from acute poison to growth inhibition, fertility, *escape*, and lethal effect (Connell and Miller, 1995). The first organismic response on pollution is detoxification through physiological processes. However, actual response is started on molecular level such as at DNA or gene level. This is occurred if the pollutant accumulated either on water, sediment or soil. According to Mukono (2005) and Lu (1995), pollutant causes chromosome damage and DNA mutation. DNA mutation might lead to a loss or form of restriction sites when DNA cut by restriction enzymes Therefore, it is possible to detect pollution using PCR-RFLP marker such as using cytochrome

c oxidase I gene. This method is cost effective and faster than blotting and DNA microarray. Moreover, in certain cases RFLP more effective compared to DNA microarray (Smith et al*.,* 2002).

Previous studies on molecular effect of pollutant has been done, such as on plant (Jonak et al., 2004; Cardinale et al., 2000; Cakmak and Marschner, 1991; Goldstein et al., 1988), animal (Mouches et al., 1987), coral (Hashimoto et al., 2004) and microbe (Park et al., 1995). All studies used a gene which directly involved on detoxification, e.g. malate dehydrogenase gene (Park et al., 1995), ATP surphurylase gene (Pilon-Smits et al., 1999), *merA* gene (Nazaret et al., 1994) *merB* gene (Bizily et al., 1999), mercuryreductase gene (Ogunseitan, 1998), collagen type II gene (Li et al*.,* 2006), H43 gene (Rubinelli et al., 2002), and superoxide dismutase gene (Roberts et al., 2006). Those studies observed gene expression using *blotting* and *DNA microarray*, whereas no study has been done on effect of pollutant on nucleotide level, especially on nucleotide sequences of a gene that is not involved on detoxification such as mitochondrial gene. A study showed the effect of pollutant on mitochondrial gene activities (Gonzalez et al., 2006). Craig et al., (2007) note that pollutant, especially cooper, affecting the expression of cytochrome gene.

Cytochrome gene is consist of three type of gene that is cytochrome c oxidase 1 (COI), cytochrome c oxidase 2 (COII), and cytochrome c oxidase 3 (COIII) gene. As genetic marker COI has some advantages, i.e. it has restriction sites (Arnaud et al., 2000; Arnaud et al., 2003; Lam and Morton, 2003; Arnaud et al., 2005), and has high mutation rate (Bucklin et al., 1999). High mutation rate of COI gene is suggested that it will easily mutate if the organisms is exposure to polluted ecosystem and shows differences to that isolated from an organism inhabit unpolluted ecosystem if those cut by restriction enzymes.

Mud clams, also known as mangrove clams *Polymesoda erosa* (Solander, 1786) is widely distributed across Indo-Pacific coastal regions, including in the Segara Anakan Cilacap. Adult individual of mangrove clams *P. erosa* (Solander, 1786) might reach 11 cm in maximum shell size (Gimin et al., 2004). Therefore, the clams are commonly consumed by local community who lives closed to the coastal region (Meehan, 1982) including the community near the Segara Anakan areas (Dudley et al., 2000).

This clam is a sessile organism which might face adverse effect of environmental alteration due to input of pollutant. Mud clam was chosen as an object of this study because of several reason, such as it is commonly used as bio-indicator for pollution, capable to accumulate pollutant on their tissue without kill them, abundant, long life span, big size, easy to handle, tolerant to brackish- and freshwater, and show positive correlation between pollutant concentration on environment and on body tissue (Philips, 1980). Their capability to accumulate heavy metal pollutant on their tissue is suggested due to it has high adaptation on their genetic constituent.

The aims of this study were: 1) development of RFLP fragment of COI gene from *P. erosa* (Solander, 1786) using restriction endonuclease enzymes and 2) characterize *P. erosa* collected at two different habitats using COI-RFLP markers.

MATERIAL AND METHODS

Survey method was used during the study by applying purposive random sampling. Samples were collected at five sampling sites which was consists of one site at Donan River and five sites at Segara Anakan (Figure 1). Tissue samples were cut off from muscle tissue with the help scissor and forceps. Tissue samples were preserved in ethanol 96% and stored at room temperature. Genome total was isolated using Chelex method following protocol from Walsh et al. (1991) with small modification (Nuryanto and Kochzius, 2009). Fragment of COI gene was amplified using PCR technique with a pair of universal primer from Folmer et al. (1994). PCR products were cut off by ten restriction enzymes, i.e. *Alu*1, *BamH*1, *BsuR*1, *Hpy*31, *EcoR*1, *Hae*III, *Hind*III, *Hinf*1, *Rsa*1, and *Vsp*1.

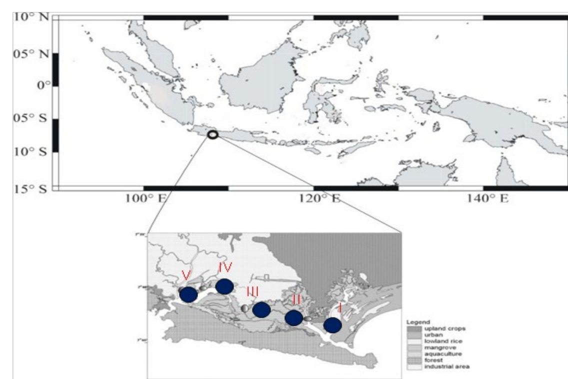

Figure 1. Sampling sites

Selected enzyme was determined descriptively based on its ability to cut PCR products. Nucleotide mutation and molecular marker was also analyzed descriptively according to the appearance of restriction fragment on agarose gel. Haplotype diversity *h* (Nei, 1987) and nucleotide diversity π (Nei and Jin, 1989) was estimated with the help of Arlequin software (ver. 2.0; Schneider et al., 2000). Null hypothesis of neutral evolution of used marker was tested by Tajima's *D* test (Tajima, 1989) and Fu's *Fs* test (Fu, 1997) with 10,000 permutations as implemented on Arlequin program (ver. 2.0; Schneider et al., 2000).

RESULTS AND DISCUSSION

Development of RFLP Markers

Restriction fragmant length polymorphism (RFLP) markers were developed by applying restriction endonuclease enzymes on PCR products. Previous development was using ten restrictions enzymes. However, only two (*Alu*1 and *Vsp*1) out of the ten enzymes resulted RFLP markers, whereas the rest of eight enzymes were not.

Therefore, the obtained RLFP markers resulted by *Alu*1 and *Vsp*1 enzymes were used for further molecular characterization of *P. erosa* (Solander, 1786) collected at two different habitats.

This result was different to several previous studies which all used the same enzymes on other species of bivalve. All the ten enzymes could cut COI fragment and resulted polymorphic sites such as on *Pteria sterna* (Arnaud et al., 2005), *Pinctada margaritifera* (Arnaud et al., 2003), *Biomphalaria* (Vidigal et al., 2002), *Pinctada mazlantica* (Arnaud et al., 2000), *Calyptogena soyoae*, *C. Solidissima* dan *C. Fausta* (Kojima et al., 1994). This proves that each species has different or show variation on their COI gene, so it will show different pattern of RFLP markers if it is exposured on the same restriction enzymes.

There are two possibilities that RFLP marker was not resulted in this study: 1) naturally there were lack of restriction sites on the COI gene of *P. erosa* from Segara Anakan and 2) the amplified COI gene is suggested undergo mutation event due to heavy metal pollution on sediment and water at sampling sites. This mutation is suggested lead to a loss of restriction sites and therefore no RFLP marker was resulted when the COI fragment was exposure to restriction enzymes. Mukono (2005) and Lu (1995) had noted that pollutant might cause chromosomes damage and DNA mutation. DNA mutation form and loss of restriction sites resulted by the alteration of fragment number or DNA length which cut off with restriction enzymes

Molecular Characterization of *Polymesoda erosa*

Molecular characterization of *P. erosa* was performed using RFLP maker resulted from application of *Alu*1 and *Vsp*1 restriction enzymes on COI PCR product. *P. erosa* tissues samples were collected at five sampling sites with different Pb content in their sediments. According to their Pb content, those five sites could be grouped into two different habitats i.e. heavy polluted and less polluted habitats. Sampling sites number I, II, III, and IV were less polluted (Pb concentrations range from 18.26 to 38.14 mg/kg), whereas station V was heavily polluted (Pb content of 124.40 mg/kg).

Six different RFLP markers were obtained from *Alu*1 application, i.e. RFLP marker with the size of 300, 350, 400, 450, 500, and 600 bp (Figure 2). It can be seen from the Figure 2 that RFLP markers was obtained from almost all *P. erosa* samples collected at less polluted. However, some of samples from less polluted and all samples from heavy polluted habitat did not result RFLP markers.

The exposures of COI PCR product on *Vsp*1 restriction enzyme obtained three RFLP markers concist of 100, 250, and 400 bp fragments (Figure 3). Similar to *Alu*1 application, using *Vsp*1 enzyme, RFLP markers were also resulted from almost all samples collected at less polluted habitat. Some of samples from less polluted habitat and all samples from heavy polluted habitat did not result RFLP markers. These results prove that *P. erosa* collected at less- and heavily- polluted can be differentiate using COI RFLP markers.

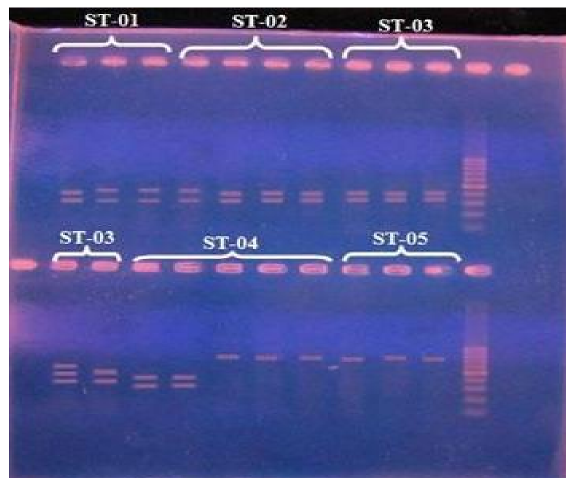

Figure 2. Amplified PCR product cut off with *Alu1* restriction enzyme

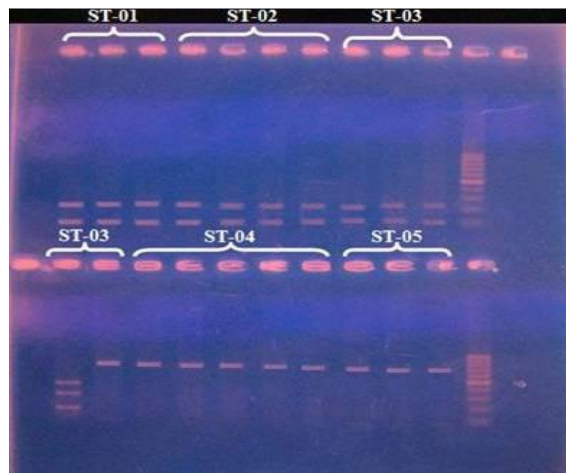

Figure 3. Amplified PCR product cut off with *VspI* restriction enzyme

The phenomena could be explain at least by two arguments. Firstly, there would be two allele of COI gene occurred on *P. erosa* (Solander, 1786) in Segara Anakan. The expression of these two alleles is suggested affected by environmentals factors, in particular is Pb concentration on sediment. Significantly differences in Pb concentrations at two different habitats in Segara Anakan lead to different expression of COI gene of *P. erosa* as shown in different PCR-RFLP markers were found. Less polluted habitat is characterized by the presence of RFLP markers, while hevaily polluted habitat was characterized by no RFLP fragment. This finding was agreed with what Brown (1999) had noted that one allele will shows precise sequences for restriction site so it will be cut if it is treated with restriction enzyme and result RFLP fragments. Sequence of the other allele has altered, therefore the restriction site no longer known by restriction enzyme. In the second case, two fragments of DNA will still in vicinity although cut off by restriction enzyme and RFLP fragment is obtained.

Tabel 1. Hasil pengukuran logam berat dari lima lokasi penelitian dan baku mutu air

Media	Jenis Logam Berat	Stasiun I	Stasiun II	Stasiun III	Stasiun IV	Stasiun V	Baku Mutu Air Kelas II PP 82/2001
				Satuan mg/l			
Air	Cd	0,020	0,010	0,010	< 0,005	0,029	max 0,001
	Hg	< 0,001	< 0,001	< 0,001	< 0,001	< 0,001	max 0,002
	Pb	0,321	0,408	0,346	0,304	0,473	max 0,003
				Satuan mg/kg			
Sedimen	Cd	< 0,005	< 0,005	< 0,005	< 0,005	< 0,005	
	Hg	< 0,001	< 0,001	< 0,001	< 0,001	< 0,001	
	Pb	18,26	19.27	38,14	23,03	124.40	

Secondly, if we come into detail about the level of heavy metal pollution, particularly for Pb concentration on sediment, station V (heavily polluted habitat) has Pb concentration as high as 124.40 mg/kg. At the same time, Pb concentrations in sediment at four other stations (less polluted habitats) were less than 40 mg/kg. However, these concentrations proved that all areas of Segara Anakan are polluted by Pb. The present of RFLP *Alu*1 fragment on the samples from less polluted habitat and the absent of this fragment at heavily polluted habitat had proven that heavy metal pollution might cause nucleotide mutation on cytochrome c oxidase I gene of *P. erosa* and led to the lost of restriciton sites on that gene. Mutation is suggested as adaptation processes to response on high concentration of heavy metal. According Mukono (2005), organism response to pollutant through idiosincration reaction, mean abnormal genetic reaction.

The result of this study has indicated that PCR-RFLP of COI gene resulted from application of *Alu*1 and *Vsp*1 restriction enzymes can be used as molecular character on *P. erosa* inhabit Pb polluted ecosystem. However, the sensitivity of this technique needs to be further evaluated to obtain reliable and valid technique for direct application. Moreover, it has to be checked also the level of Pb which can already cause DNA mutation.

CONCLUSION

It can be concluded that 1) RFLP markers of cytochrome c oksidase 1 gene can be obtained from *P. erosa* collected at Segara Anakan Cilacap using *Alu*1 and *Vsp*1 restriction enzymes and 2) COI-RFLP markers of *P. erosa* from Segara Anakan Cilacap can be used as molecular character to differentiate *P. erosa* inhabiting less Pb polluted and heavily Pb polluted habitats.

ACKNOWLEDGEMENTS

We would like to thank to the institutions and individuals that have made our study possible: Jenderal Soedirman University (Unsoed, grant no. 1891.1/H23.9/PN/2011), which funded the project; LPPM Unsoed for project co-ordination; The Dean of Biology Faculty of Unsoed for the permission; fisherman at Segara Anakan Cilacap for help during field work; Kusrinah for help during laboratory works.

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