

Genetic variation of the mangrove species *Avicennia marina* in heavy metal polluted estuaries of Cilegon Industrial Area, Indonesia

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Abstract. Manurung J, Siregar IZ, Kusmana C, Dwiyanti FG. 2017. Genetic variation of the mangrove species *Avicennia marina* in heavy metal polluted estuaries of Cilegon Industrial Area, Indonesia. *Biodiversitas* 18: 1109-1115. Mangrove forests are often facing various anthropogenic disturbances, particularly in the industrial area. However, information on the status of industrial heavy metal pollution and its impact on the genetic variation of mangrove species, such as *Avicennia marina*, particularly in Indonesian industrial area has not been assessed. In this study, the classification of heavy metals in two estuaries of Cilegon Industrial Area (Pelabuhan Warna Sari and Krakatau) and one estuary outside Cilegon Industrial Area (Pulau Sangiang) were determined by Sediment Quality Guidelines-States Environment Protection Agency (SQGs-USEPA). Furthermore, the genetic diversity of 72 individuals of *A. marina* in the three estuaries were examined using four microsatellite loci (M3, M64, M81 and M98). The results showed that Krakatau area was the heavily polluted estuary based on SQGs-USEPA and have a lower genetic variation ($H_e = 0.54$) than Pelabuhan Warna Sari (moderately polluted, $H_e = 0.56$) and Pulau Sangiang (non-polluted, $H_e = 0.60$) suggesting a notable of deficiencies of genetic variation of *A. marina* in heavy polluted estuary compared to moderately and non-polluted estuary. Considering the low level of genetic variation in heavily polluted area, transplantation among estuaries and introduction of propagules from non-polluted estuary or other regions are needed as one of the conservation efforts.

Keywords: *Avicennia marina*, estuary, genetic variation, heavy metal, industrial area

INTRODUCTION

Heavy metal pollution is one of the most worldwide environmental problems that cause toxicity to organisms at various levels of the tropic (Wang et al. 2010). In the case of plant, toxicity of heavy metal pollution accompanied by its physical disturbance can influence plant survivorship, recruitment, reproductive success, mutation rates, and migration and consequently affect the genetic diversity of exposed populations (Deng et al. 2007). However, green plants through their various mechanisms have an enormous ability also to absorb pollutants from the environment and accomplish their detoxification (Ali et al. 2013), by involving their intricate mechanism in gene regulatory system (Grativol et al. 2012), which known as hyperaccumulator. Some of them are potential for the phytoremediation of heavy metal-contaminated land (Pulford and Watson 2003).

Residual of industrial and urban are discharged through rivers that eventually reach the mangrove forests and cause negative impacts on the presence of organisms and quality of the environment (Oliveira et al. 2014). At the same time, mangrove ecosystem has a vital role in the marine ecosystem in relation to nutrient cycling, absorption of pollution, coastal zone protection from natural disasters,

increasing productivity and diversity of organisms (Kathiresan and Bingham 2001) and can be used as indicator of coastal change (Blasco et al. 1996). *Avicennia marina* is ecologically important mangrove pioneer species widespread in the subtropical and tropical regions in various types of tidal and wide range of salinity tolerance and temperature (Duke 1991), its roots have the ability to withstand sediment (Noor et al. 2006) and has unique ecophysiological response to trace metal contamination (Naidoo et al. 2013). Recently, this species has been reported as potential metal bioaccumulator (Parvaresh et al. 2011; Usman et al. 2013; Chaudhuri et al. 2014). Another investigation also reveals that *Avicennia sp.* is the most tolerant species respects to heavy metal compared to other mangrove species due to bioaccumulation potential (Maiti and Chowdhury 2013).

Studies on the industrial waste of various heavy metals accumulated in the sediments of mangrove forests in various countries have been widely investigated. The toxicity, growth and accumulation relationship of heavy metal pollution to *A. marina* in the contaminated environment (sediment) has been previously investigated (MacFarlane 2002; MacFarlane and Burchett 2002; MacFarlane et al. 2003; Usman et al. 2013). In those studies, sediment quality criteria were evaluated by the

United State Environmental Protection Agency (USEPA), which has been used worldwide (Burton 2002) and Sediment Quality Guidelines (SQGs) have been used as a reference in determining the level of contamination on the location of the research that has been previously applied by Luo et al. (2010) and Usman et al. (2013). The study on sediment characteristics, metal levels, tree attributes, leaf morphology and genetic variation using isozyme in *A. marina* in three estuaries of Sydney (Australia) has also been investigated (Melville and Burchett 2002). However, a study on the genetic diversity of *A. marina* particularly distinguished as potential metal bioaccumulator in sediment contaminated heavy metals by industrial activities in Indonesia is still not yet carried out. Therefore, the aims of present study were (i) to classify the level of pollution of various heavy metals in sediments of mangrove forests in Cilegon Industrial Area, Indonesia based on SQGs standard, and (ii) to assess genetic diversity of mangrove species, *A. marina* in the most industrialized area of Indonesia. It is expected that the results of this research will be useful for determining policy and species conservation strategies and rehabilitation of mangrove in specific areas contaminated by heavy metals.

MATERIALS AND METHODS

Study area

The research was conducted from October 2014 to April 2015. Sediments and leaf samples were collected from three natural mangrove forests in Banten province, Indonesia (Figure 1). The two sites were located in Cilegon Industrial Estate of Indonesia and close to industrial activities, which considered as polluted mangrove forest by heavy metal, namely Krakatau ($5^{\circ}59'48.4''$ S - $105^{\circ}59'0.4''$ E) and Pelabuhan Warna Sari (Pelabuhan WS) ($5^{\circ}59'4.9''$ S - $105^{\circ}59'25''$ E). Whereas, one site was located outside Cilegon Industrial Estate of Indonesia and far away from industrial activities, which chosen as the non-polluted mangrove forest, namely Pulau Sangiang ($5^{\circ}57'40.9''$ S - $105^{\circ}51'11.2''$ E). The sample of sediment was analyzed in the Laboratory of Soil Research Institute, Cimanggu Bogor, West Java, Indonesia, while DNA analysis was carried out in Laboratory of Forest Genetics and Molecular Forestry, Department of Silviculture, Faculty of Forestry, Bogor Agricultural University (IPB), Bogor, West Java, Indonesia.

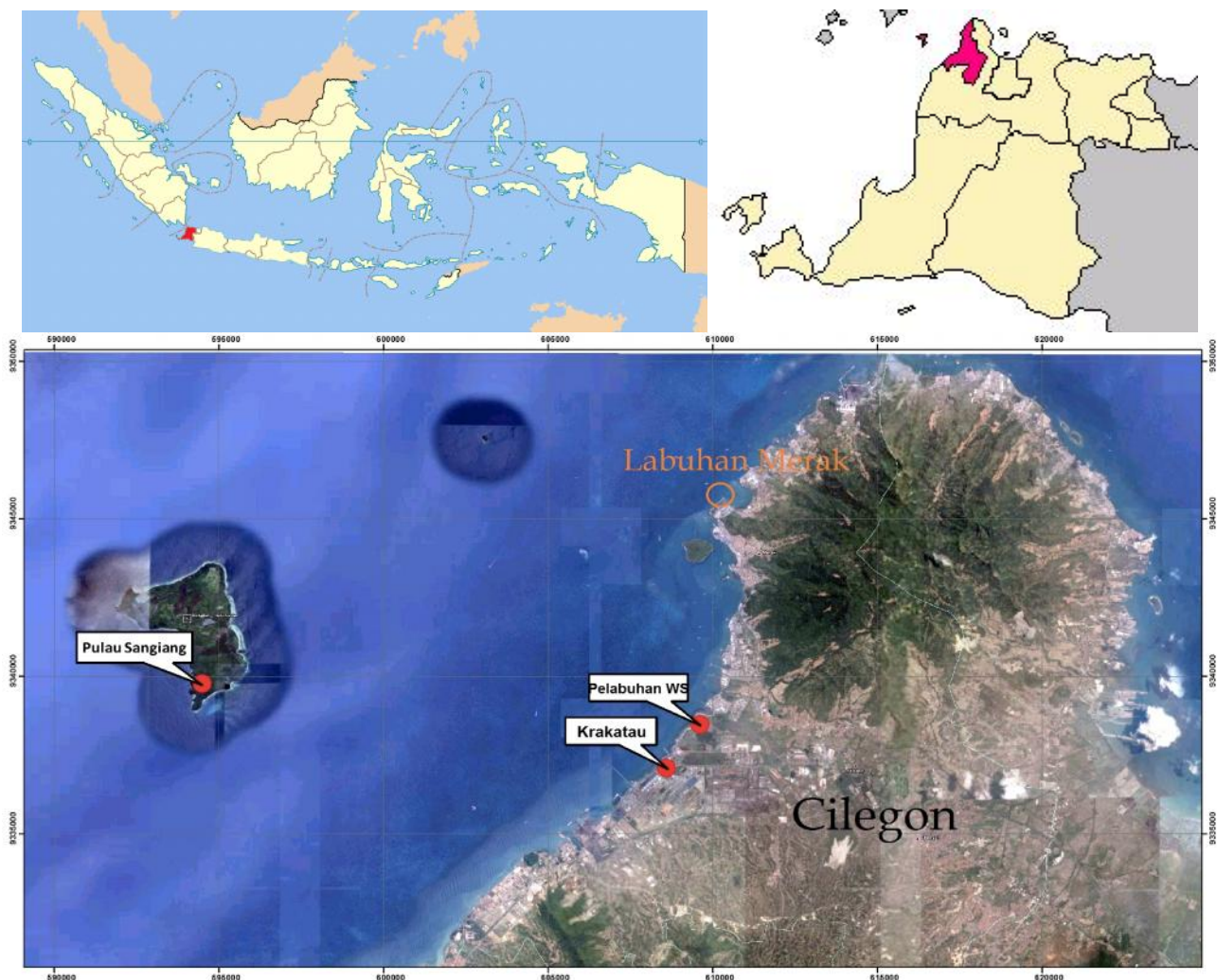


Figure 1. Sampling location of *A. marina* in Banten Province, Indonesia

Procedures

Sediment heavy metals analysis

The collected sediments from surface of three sites were sampled randomly at a depth of 0-15 cm with more than 10 composite samples of at least 1 kg of each site following to Usman et al. (2013) with some modification. The total content of 16 heavy metals (Pb, Cu, Zn, Cr, Ni, As, Cd, Ag, Se, Sn, Co, No, Fe, Al, Mn, and B) in sediment was digested by Atomic Absorption Spectrometer (AAS) method with extraction using HNO₃ and HClO₄ (Eviati and Sulaiman 2009). The classification of each heavy metal determined by SQGs-USEPA that has been developed around the world (Luo et al. 2010; Usman et al. 2013).

DNA extraction and microsatellite genotyping

The 24 individuals fresh leaves of *A. marina* were randomly collected from the regeneration trees at each estuary for genetic analysis. In total, 72 individuals were used in this study. Total genomic DNA was extracted using modified CTAB method (Doyle and Doyle 1990) and visualized in 1 % of Agarose gel electrophoresis (Weising et al. 2005; Doyle and Doyle 1990).

Four microsatellite loci (M3, M64, M81, and M98) developed for *A. marina* by Maguire et al. (2000a) were used in this study to characterize the genetic diversity. Polymerase Chain Reaction (PCR) was performed with a 14 µL reaction volume containing 2 µL Nuclease-Free Water, 2 µL of each Forward and Reverse primer and 8 µL GoTaq® Green Master Mix 2X using AB Applied Biosystem Verity™ Thermal Cycler (<https://www.appliedbiosystem.com>). Electrophoresis for examining PCR products was performed using EC3000P series 90 programmable vertical electrophoresis unit, following the procedure described by Wang et al. (2009). The 1 % Polyacrylamide gel was stained with silver nitrate stain and photographed under a UV transilluminator in order to visualized PCR products (Creste et al. 2001; Benbouza et al. 2006).

Data analysis

The GPS coordinates of three estuaries were analyzed using ArcGIS 10. While, the genetic data analysis was based on the scoring results of the DNA bands in the polyacrylamide gel. Then, basic statistics of genetic diversity, including number of allele (N_a), effective number of allele (N_e), percentage locus of proportion of polymorphic loci (P), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (F), were calculated using PopGene 32 Version 1.31 (Yeh et al. 1999). F -statistics (F_{is} , F_{it} and F_{st}) by Hartl and Clark (1989) were estimated using PopGene 32 Version 1.31 (Yeh et al. 1999). Analysis of Molecular Variance (AMOVA) that showed alleles distribution in each locus, within and among population, were calculated using GenAlex Version 6.5 (Blyton and Flanagan 2006).

Nei's genetic distances (Nei 1978) were used to construct UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram, which showed the population clustering based on genetic distance (Hartati et al. 2007) associated with geographic distance, using NTSys

version 2.0 (Rohlf 2008).

RESULTS AND DISCUSSION

Total concentration of heavy metals in sediments and Sediment Quality Guidelines (SQGs)

The present study revealed that 9 of 16 heavy metals analyzed, i.e. Pb, Cu, Zn, Cr, Mo, Fe, Al, Mn, and B were found in sediments of three estuaries, with Copper (Cu) becomes the most metals that contaminate the three locations (Table 1). The certain source of Cu that contaminated these region is still unknown because industries discharge their waste to the same estuary namely Cilegon Industrial Area.

Two of 16 heavy metals found at one or two estuaries only, such as, Tin (Sn) in Krakatau and Cobalt (Co) in Pulau Sangiang and Pelabuhan WS. Whereas, other five pollutants (Cd, Ni, As, Ag, Se) were not detectable in all estuaries with their detection limit (data not published). However, a standard guideline for other metal contaminants (Sn, Mo, Fe, Al, and B) need to be developed for future investigation of determining pollution level.

Among the studied sites, the highest concentration of Pb (59 mg kg⁻¹), Cu (105 mg kg⁻¹), Zn (791 mg kg⁻¹), Cr (69 mg kg⁻¹), Fe (7.38 mg kg⁻¹) and Mn (0.09 mg kg⁻¹) were found in Krakatau indicated that this area was the most polluted area. Other than that, the concentration of Cu and Zn in Krakatau area were in excess of SQG indicating heavily polluted category, while the concentration of Pb and Cr were in the range of moderately polluted category (Table 1). In addition, Krakatau was the only site where Zn was found.

Sediments in Pelabuhan WS were heavily polluted by Cu (60 mg kg⁻¹) and moderately polluted by Zn (90 mg kg⁻¹) (Table 1). Therefore, Pelabuhan WS was considered as a moderately polluted estuary. Whereas, sediments in Pulau Sangiang was a moderately polluted area by Cu (46 mg kg⁻¹) and not polluted by other heavy metals observed in this study based on SQGs category suggested the light contaminant loads from industrial contribution have occurred in Pulau Sangiang. Furthermore, Pulau Sangiang was considered as a non-polluted estuary.

Population genetic variation of *A. marina*

The genetic diversity parameters are shown in Table 2. There were 14 alleles detected at four loci among 72 individuals sampled. The mean number of alleles (N_a) per locus ranged from 2.50 to 3.25, with a mean value of 2.92 per locus (Table 2). The proportion of polymorphic loci (P) was 1.00 in every population. Overall populations, average observed (H_o) and expected heterozygosity (H_e) values were 0.90 and 0.56 respectively. At the population level, the mean observed heterozygosity (H_o) of Pulau Sangiang, Pelabuhan WS and Krakatau were 0.92, 0.98, and 0.80, respectively. The highest mean of expected heterozygosity ($H_e=0.60$) was found in the non-polluted estuary, Pulau Sangiang ($H_e=0.56$), followed by moderately polluted area, Pelabuhan WS and the lowest ($H_e=0.54$) was found in the most polluted area, Krakatau (Table 2).

Table 1. Total concentration of heavy metal and classification of samples based on SQGs in collected samples of mangrove sediments

Heavy metals (mg kg ⁻¹)	Location			Standard Quality Guidelines (SQGs*)		
	Pulau Sangiang	Pelabuhan WS	Krakatau	SQG non-polluted	SQG moderately polluted	SQG heavy polluted
Pb	4	18	59	<40	40-60	>60
Cu	46	60	105	<25	25-50	>50
Zn	63	90	791	<90	90-200	>200
Cr	6	14	69	<25	25-75	>75
Ni	nd	nd	nd	<20	20-50	>50
As	nd	nd	nd	<3	03-08	>8
Cd	nd	nd	nd	-	-	>6
Ag	nd	nd	nd			
Se	nd	nd	nd			
Sn	nd	nd	108			
Co	2	5	nd			
Mo	45	40	35			
Fe	1.94	3.8	7.38			
Al	4.13	8.79	8.4			
Mn	0.02	0.05	0.09			
B	15	26	20			

Note: *SQGs from Luo et al. (2010) and Usman et al. (2013), nd: not detectable (detection limit 0.11 g kg⁻¹).

Table 2. Summary of genetic diversity parameters in three populations of *A. marina*

Populations	N	N _a	N _e	P	H _o	H _e	F
Pulau Sangiang	24	3.25	2.73	1.00	0.92	0.60	-0.59
Pelabuhan WS	24	3.00	2.31	1.00	0.98	0.56	-0.78
Krakatau	24	2.50	2.18	1.00	0.80	0.54	-0.50
Mean		2.92	2.41	1.00	0.90	0.56	-0.63
SE		0.29	0.21	0.00	0.06	0.03	0.12

Note: Number of samples (N), mean number of alleles (N_a), mean effective number of alleles (N_e), proportion of polymorphic loci (P), mean observed heterozygosity (H_o), mean expected heterozygosity (H_e), mean fixation index (F), and standard error (SE) for the four loci in three populations of *A. marina*.

Table 3. Summary of F-statistics values for each locus across three populations of *A. marina*

F-statistics	M3	M64	M81	M98	Mean	SE
F _{is}	-0.677	-0.780	-0.157	-0.924	-0.635	0.167
F _{it}	-0.516	-0.767	-0.031	-0.920	-0.558	0.195
F _{st}	0.096	0.007	0.109	0.002	0.054	0.028

Note: deficiency of heterozygosity relative to the Hardy-Weinberg expectation (F_{is}), the overall inbreeding coefficient (F_{it}), differentiation among populations (F_{st}), and standard error (SE).

Table 4. Genetic distance and geographic distance

Genetic	Geographic	Pulau Sangiang	Pelabuhan WS	Krakatau
Pulau Sangiang			15.40	14.9
Pelabuhan WS		0.161		1.53
Krakatau		0.125	0.117	

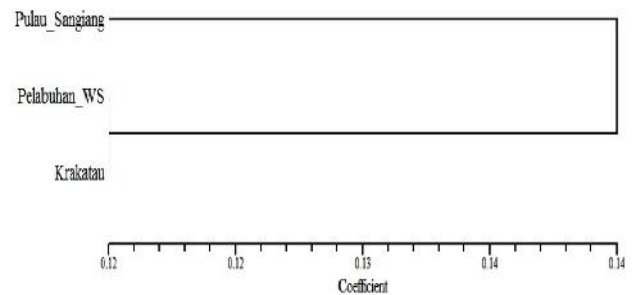


Figure 2. UPGMA Dendrogram of three populations of *A. marina*

Population genetic differentiation of *A. marina*

The genetic differentiation of *A. marina* in three estuaries was analyzed using F-statistics (Table 3). The inbreeding index (F_{is}) values were negative in all loci and ranged from -0.157 (locus M81) to -0.924 (locus M98), with a mean number of F_{is} was -0.635. The inbreeding coefficient (F_{it}) values were also negative in all loci and ranged from -0.031 (locus M81) to -0.920 (locus M98), with a mean F_{it} value, was -0.558. Furthermore, the value of genetic differentiation among populations (F_{st}) ranged from 0.002 (M98) to 0.109 (M81), with a mean number of F_{st} was 0.054.

AMOVA based on allelic distance matrix for F-statistic analysis in all populations showed that value of F_{st}, F_{is} and F_{it} were 0.082, -0.589, and -0.459, respectively. F_{st} mean value in all loci and in all population were interpreted have moderate genetic differentiation. Percentage of genetic variation from molecular variance showed that each individual in all populations that the highest variation was within the individual (95%), while among populations was 5% and there was no variation among individual (0%).

Relationship between genetic and geographical distance in *A. marina*

Microsatellite analysis of the population in three estuaries assessed to confirm that geographic distance between the estuaries is of consecutive gene exchange in this species (Table 4). Table 4 showed that the largest genetic distance (0.161) was between moderately polluted estuary (Pelabuhan WS) and non-polluted estuary (Pulau Sangiang) and the smallest distance (0.117) was between heavy polluted estuary (Krakatau) and moderately polluted (Pelabuhan WS).

Thus, the genetic distances of *A. marina* populations were concordant with geographical coastal distances in this study. This pattern is also supported by the UPGMA dendrogram, which showed that heavy polluted estuary (Krakatau) and moderately polluted (Pelabuhan WS) were in the same cluster, apart from non-polluted estuary (Pulau Sangiang) (Figure 2).

Discussion

The excess concentration of copper (Cu) and Zinc (Zn) and moderate concentration of Lead (Pb) and Chromium (Cr) based on SQG category in Krakatau compared with

other studied sites suggested that strong contaminant loads from industrial contribution have occurred in Krakatau, as this site is directly located at the mouth of stormwater discharge points. Therefore, Krakatau was considered as a heavily polluted estuary. Nath et al. (2014) also reported that the occurrence of strong metal concentration in most embayment is due to a dominant stormwater source of metal derived from residential land use and road surfaces. Furthermore, the widely occupied by Cu and moderate contaminant loads from industrial contribution in Pelabuhan WS indicated that this site was a moderately polluted estuary. Whereas, the moderately polluted by Cu and not polluted by other heavy metals in Pulau Sangiang based on SQGs category indicated that this site was a non-polluted estuary. There was some evidence that pollution factors such as sediment metal levels may be among significant local selection pressures in the different estuaries (Melville and Burchett 2002), and significant decreases in total biomass, number of leaves and plant height in *A. marina* (Naidoo et al. 2013). In the present study, although the very high concentration of Cu, Zn and Sn (Table 1) were found in one of three estuaries (Krakatau), there were no significant differences with leaves characteristic of *A. marina* in this location compared to other estuaries which were moderately polluted (Pelabuhan WS) and non-polluted (Pulau Sangiang) estuaries (Manurung 2015). This pattern indicated that sediment analysis could not reveal the effects of heavy metal pollution on the morphological characteristics of *A. marina*. Therefore, molecular approach to investigate the effects of heavy metal stress in this species is needed in this study, given that this approach is more reliable tools, which can also be used on conservation genetics implication.

Genetic variation among and within three populations of *A. Marina* was successfully revealed using four polymorphic microsatellite loci, given that microsatellite marker detected more genetic differentiation and was considerably more variable than AFLPs (Maguire et al. 2002) and RAPD marker (Hazarika et al. 2013). Overall populations showed that genetic diversity of *A. marina*, which indicated by average observed (H_o) and expected heterozygosity (H_e) values in this study were higher than genetic diversity of *A. germinans* in Colombian Caribbean (Leiva et al. 2009) suggesting that the genetic diversity in the present study was comparable with another *Avicennia* species. Meanwhile, at the population level, the highest genetic diversity was found in the non-polluted estuary, Pulau Sangiang, followed by moderately polluted area, Pelabuhan WS, and the lowest was found in most polluted area, Krakatau suggesting that the reduction of genetic diversity occurred from the non-polluted estuary to the most polluted estuary. This pattern also revealed that the levels of genetic diversity of *A. marina* studied here have been influenced by the heavy metals pollution. The reduction of genetic variation in heavy polluted area in the present study was also found in hyperaccumulator plant such as *Sedum alfredii* using RAPD marker (Deng et al. 2007) and *Deschampsia cespitosa* using ISSR and microsatellite marker (Gervais and Nkongolo 2011).

However, the results of present study was contrary to the study of *Silene paradoxa* L. using RAPD marker (Mengoni at al. 2000), which reported that genetic diversity of the uncontaminated population was the same as that of the contaminated population and the study of *Prosopis juliflora* using ISSR (Sharma at al. 2013), which reported that the reduction of genetic diversity was found in lowest contaminated population.

In the present study, F -statistics showed the presence of the high heterozygous excess in three *A. marina* populations ($F_{is} = -0.635$) and a low level of genetic differentiation among populations of *A. marina* ($F_{st} = 0.054$). This pattern may be due to environmental and ecological factors, particularly past sea level and climatic changes (Maguire et al. 2000b). Meanwhile, the largest genetic distance was between moderately polluted estuary (Pelabuhan WS) and non-polluted estuary (Pulau Sangiang) and the smallest distance was between heavy polluted estuary (Krakatau) and moderately polluted (Pelabuhan WS). The small genetic distance between Krakatau and Pelabuhan WS was expected since the distance between Krakatau and Pelabuhan WS is only 1.5 km apart. Other than that, the phase of dispersal in the life history of *A. marina* is relatively short due to cryptoviviparous propagules, which is contain a well-developed embryo (Clarke 1993). This genetic distance pattern was also supported by the UPGMA dendrogram, which showed that heavy polluted estuary (Krakatau) and moderately polluted (Pelabuhan WS) were in the same cluster, apart from non-polluted estuary (Pulau Sangiang). This pattern indicated that microsatellite analysis of the population in three estuaries assessed to confirm that geographic distance between the estuaries was of consecutive gene exchange in this species. Geographic distances correspond to gene flow and dispersal potential of propagules for widespread mangrove species (Duke et al. 1998), and influence the limitations in genetic difference of *A. marina* (Melville and Burchett 2002).

This study successfully revealed genetic variation and distance of mangrove species *A. marina* in three estuaries with the different level of heavy metal pollution. Genetic variations corresponded with level of pollution. Deficiencies of genetic variation were occurred in heavy polluted estuary compared to moderately polluted and non-polluted estuaries. The genetic distances of *A. marina* populations corresponded with geographical coastal distances, which the largest genetic distance was between moderately polluted estuary and non-polluted estuary and the smallest genetic distance was between heavily polluted estuary and moderately polluted estuary. In order to increase the genetic variation in the heavy polluted estuary, future transplantation plans should introduce propagules or saplings from non-polluted estuary or other regions, which having the different genetic structure. Additional works with a wide variety of mangrove species and development of other heavy metals assessment standard are recommended for a more comprehensive understanding of mangrove plant as phytoremediation.

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