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Research Article

Genetic Diversity Analysis of *Rhacophorus margaritifer* (Schlegel, 1837) in Baturraden, Purwokerto, Central Java, Indonesia Revealed by Based on RAPD Marker

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ABSTRACT
Rhacophorus margaritifer is an endemic species of arboreal frog in Java. Previous studies found this frog in several locations of Baturraden, namely in primary forest of Ketenger area, along Pancuran Pitu tourism track, and Baturraden Botanical Garden. There were still limited studies of molecular diversity of *R. margaritifer* and no prior data from population in the southern slope of Mount Slamet. This study aimed to look at the genetic polymorphism and determine the locus diversity of *R.margaritifer* population in Baturraden by using the PCR-RAPD technique. Frog tissue samples were taken from three populations in Kalipagu hiking trail (HPK 01), Baturraden hiking trail (HPK 02), and Baturraden Botanical Garden (KRB). DNA was extracted using the Chelex method. Molecular characterization was performed based on RAPD markers. The RAPD marker band pattern was changed to binary data 0-1 and analysed using Arlequin software ver.3.5. A total of 19 frog individuals were obtained during sampling at three locations. High genetic diversity had been observed in all populations with gene diversity range from 0.9643 in HPK 01 population to 1.0000 in both KRB and HPK 02 populations. A high locus variation was also observed for all populations with values of 0.159524 in KRB; 0.165816 in HPK 01; 0.192857 in HPK 02, respectively. AMOVA indicated no genetic difference among populations of *R. margaritifer* (p=0.50244).

Keywords: Baturraden, genetic diversity, RAPD, *Rhacophorus margaritifer*

INTRODUCTION
Rhacophorus margaritifer is a frog of Rhacophoridae family. It is an endemic species in Java and distributed throughout West Java to East Java (Iskandar, 1998). Previous studies found this species in several locations in the southern slope of Mount Slamet, namely in primary forest of Ketenger, Baturraden (Riyanto, 2010), Pancuran Pitu tourism track (Puspitasari *et al.*, 2017), and thematic area of Baturraden Botanical Garden (Avani, 2018).

There were still limited studies of molecular diversity of *R. margaritifer*. There is no prior data from *R. margaritifer* populations in the southern slope of Gunung Slamet, including Baturraden area. The information on species molecular diversity plays an important role in species conservation and can be used to determine specific conservation method (Funk *et al.*, 2012; Angulo & Icochea, 2010; Loeschcke *et al.*, 1994; Schonewald-Cox *et al.*, 1983). Molecular characterization of *R. margaritifer* enables to identify any diversity from individuals with the similar morphology. Molecular diversity can be discovered by detecting DNA polymorphism using Random Amplified Polymorphic DNA technique. RAPD detects DNA polymorphism based on PCR (Polymerase Chain Reaction). The RAPD primer randomly amplifies a compatible sequence of DNA genome to produce various fragment lengths (Telles *et al.*, 2006). RAPD is widely used since it is relatively fast, simple, and affordable (Anggereini, 2008; Williams *et al.*, 1990). RAPD also helps to identify genetic variation in an animal. Snell & Evans (2006) used RAPD to differentiate the larva of *Rana arvialis* and *Rana temporaria* that were morphologically similar. Padhye *et al.* (2012) reported variations on the population of *Hylarana malabarica* in northern

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Figure 1. *Rhacophorus margaritifer* (Schlegel, 1837)

West Ghat, India based on RAPD markers. Therefore, this study aimed to look at the genetic polymorphism and determine the locus diversity of *R. margaritifer* population in Baturraden using the PCR-RAPD technique. No genetic data are available on Amphibian in Baturraden, Purwokerto, Central Java, Indonesia. Therefore, it is the first report about genetic diversity of Amphibian from that area with special emphasis on *R. margaritifer*. The informations obtained in this study are vital to sustaining *R. margaritifer* since that species plays an important role in Baturraden ecosystem.

MATERIALS AND METHODS

Materials

Materials used in this study were *Rhacophorus margaritifer* (Figure 1), aquadest, 70% ethanol, absolute ethanol, GPS, headlamp, flashlight, nets, specimen bag, caliper, stationary and camera for the field sampling. Chelex 5%, Dithiothreitol (DTT), and proteinase-K for DNA isolation; Nuclease Free Water/ddH_2O, Buffer PCR, $MgCl_2$ solution, dNTPs mix, Taq polymerase, DNA Template, and RAPD primers for amplification RAPD marker. TAE 1X solution, agarose, aquadest, Ethidium Bromide (EtBr), loading dye, and DNA ladder for DNA visualization. Freezer, micropipette and tips, thermomixer, centrifuge, electrophoresis apparatus, analytical scale, UV transilluminator, beaker glass, Erlenmeyer, microwave, 0.2 mL & 1.5 mL microtube, thermal cycler (PCR), parafilm paper, gloves, vortex, scissors, tray and comb, sprayer, microtube rack, label, and phone camera for genetic diversity analysis.

Methods

This study was done from February to July 2019 in the Baturraden subdistrict, Banyumas. Sampling was carried out at the Baturraden Botanical Garden (KRB), Kalipagu Hiking Trail (HPK 01) and Baturraden Hiking Trail (HPK 02) (Figure 2). Frog samples of *Rhacophorus margaritifer* (6 samples from KRB, 8 samples from HPK 01, 5 samples from HPK 02) were obtained using a Purposive Sampling Technique. Digiti web and flap tissues were cut and stored in absolute alcohol.

DNA extraction used the Chelex method (Walsh *et al.*, 1991). The materials used for extraction were 100 µL Chelex 5%, 5 µL DTT 0.1 M, 4 µL Proteinase K, and *R. margaritifer* tissue. The mixture of solution and tissue was incubated at 56°C 1,000 rpm for 4 hours followed by centrifugation of 13,000 rpm 2 min to separate DNA from other components. At the end, it was re-incubated at 95°C for 10 min to inactivate the Proteinase enzyme.

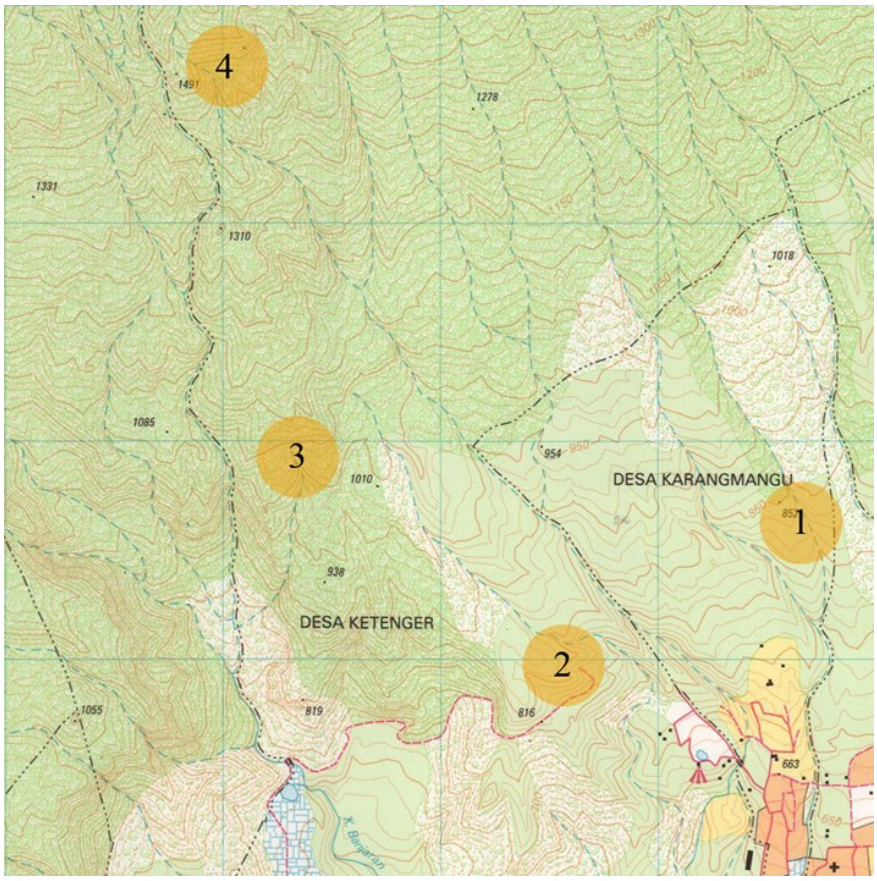

Figure 2. Sampling locations; 1. Baturraden Botanical Garden (KRB); 2. Pancuran Pitu; 3. Kalipagu Hiking Trail (HPK 01); 4. Baturrraden Hiking Trail (HPK02).

Diversity analysis was evaluated by RAPD markers amplification (Williams *et al*., 1990). The PCR composition was 17,875 µL ddH2O, 2.5 µL reaction buffer 10X, 1.5 µL MgCl2 50 mM, 1 µL dNTPs 10 M, 1 µL RAPD Primer, 0.125 µL Taq Polymerase, and 1 µL DNA template. The PCR-RAPD program ran at Pre-Denaturation 95°C for 2 min, (Denaturation 95°C for 35 s, Annealing 39/41° C for 35/40 s, Extension 72°C for 1 min repeated 35 cycles), Final Extension at 72°C for 5 min, and storage at 8°C for 5 min. The primers used were selected from 26 primers to choose the 5 best primers, namely OPB 19, OPAH 04, GEN 23, OPA 08, and OPAH 02.

The PCR-RAPD results were evaluated with 2% agarose gel electrophoresis in TAE 1X solvent. The RAPD markers pattern were changed to binary data 01 based on the presence or absence of amplified loci. Intra-population analysis is based on gene and locus diversity while inter-population analysis is based on AMOVA (Analysis Molecular of Variance) in the Arlequin ver. software. 3.5 (Excoffier & Lischer, 2010).

RESULTS AND DISCUSSION

A total of 19 individuals of *R. margaritifer* individuals were obtained from 3 locations; 8 individuals from Kalipagu Hiking Trail (HPK 01), 5 individuals from Baturraden hiking trails (HPK 02), and 6 individuals from Baturraden Botanical Garden (KRB). The distance range among the three locations was 1.725 – 3.125 km. Snout-Vent Length (SVL) of the frog samples were ranged between 35 – 60 mm. The frog populations in three locations had noticeable morphological differences. Some individuals had white dots on the dorsal and vague patterns on the dorsal and hind limbs. Hoffman & Blouin (2000) stated that many anurans showed polymorphism of color and pattern on the dorsal part of the body.

Visualization of the DNA extractions showed that the DNA genomes of *R. margaritifer* were successfully extracted from the web tissues using the Chelex method. DNA genome of samples J6, J7, J8, J9, 10, and J11 were rather vague; it was assumed that the DNA was absorbed inside the gel prior the electrophoresis. Another possibility was the low quality of the DNA during four months of storage. DNA genome of the frog was shown as smears, which was DNA with similar length and indistinct. Nuryanto *et al.* (2012) stated that smears resulted from DNA fragmentation during tissue preservation and physical treatment during DNA extractions. However, DNA smears resulted from extraction with Chelex method can be used for RAPD marker amplification, as stated in the previous studies in *Penaeus monodon* (Prastowo *et al*., 2009), *Polymesoda*

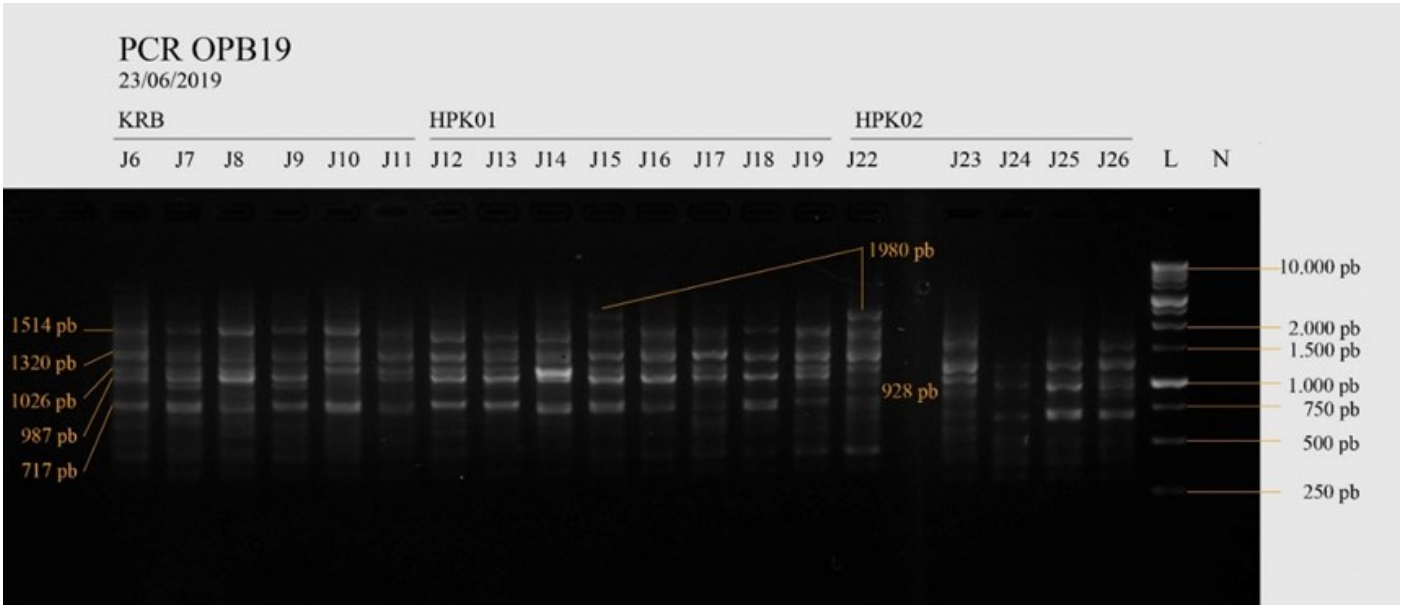

Figure 3. Visualization Results of PCR-RAPD using OPB 19 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK 01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

erosa (Nuryanto & Susanto, 2010), catfish (Nuryanto *et al*., 2012), and *Osphronemus goramy* (Khairunisa, 2015).

Selection was done in total 26 RAPD primers, OPA 01, OPA 02, OPA 03, OPA 04, OPA 05, OPA 06, OPA 07, OPA 08, OPA 09, OPA 10, OPA 11, OPA 20, OPB 01, OPB 02, OPB 19, OPAC 14, OPAH 01, OPAH 02, OPAH 04, OPAH 08, OPAH 09, GEN 11, GEN 12, GEN 13, GEN 14, and GEN 23. Several extracted genomes, J5, J7, J8, and J9, were used as templates. J5 was a sample collection of previous studies in the Laboratory of Animal Taxonomy, Jenderal Soedirman University. Every primer used for PCR was equipped with a negative control. RAPD primers were selected based on the ability to amplifying the best RAPD bands.

PCR result of primer selections showed that despite different expressions of the bands, almost all primers could amplify the DNA sample of *R. margaritifer*, except GEN 13. Primers that gave the best results were OPAH 02, GEN 23, OPA 01, OPA 05, OPAH 04, OPA 08, OPB 19, GEN 14, and OPAH 08. This result indicated several complementary sites within *R. margaritifer* DNA genome with the RAPD sequences. RAPD primers attached to the different complementary sequences of DNA template and generated various bands. Different results of bands pattern on each primer referred to specific amplification of the DNA genome. RAPD primers were specifically amplified segments on DNA templates that had the complementary sequence with the primer. Every amplified segment was considered as locus. Locus was the result of primer amplification attached on opposite 3' end template. The number and size of the bands depended on the number of primer attachment sites along with the DNA template (Williams *et al*., 1990; Kumar & Gurusubramanian, 2011).

Primer selection meant to select the primers that were capable to produce polymorphic, uncontaminated band patterns since the primers had been used eight to ten years. Evidence of contamination showed by the visualization of amplification product stained with ethidium bromide. If the negative control had shown amplified bands, then it had been contaminated. Contamination occurred on primers OPB 01, OPAC 14, OPAH 02, OPA 09, OPA 02, OPA 04, OPA 10, OPA 11, OPA 20, OPAH 09, GEN 11 and GEN 13. Contamination might be originated from the environment, reagent, or PCR operator (Hu, 2016). New reagents might be required if the reagent were contaminated. Best 5 amplified primers were randomly selected, namely OPB 19, OPAH 04, OPA 08, GEN 23 and OPAH 02. Primer OPAH 02 was selected despite the contamination since it was assumed as the result of environment and operator contamination.

There were 7 loci in the electrophoresis result of PCR-RAPD using OPB 19 primer (Figure 3). The lengths of the loci were 1980 bp, 1514 bp, 1320 bp, 1026 bp, 982 bp, 928 bp, and 717 bp. Locus allele 1980 bpb was present in sample J5 and J22, while locus allele 928 bp was present in sample J7, J9, J19, J22, J23, and J24. Locus 1320 bp and 717 bp had conserve alleles that were amplified in all samples. Meanwhile, locus allele 1026 bp was absent in sample J18, J25 and locus allele 982 bp was absent in sample J10, J22, J23.

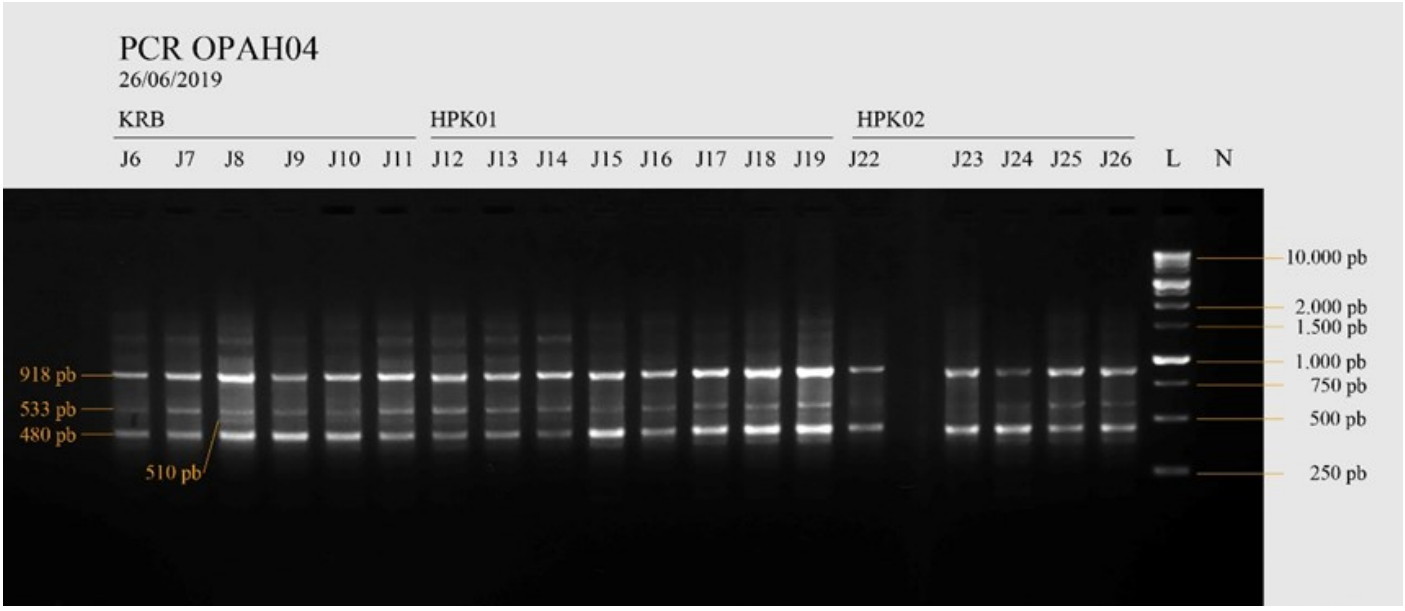

Figure 4. Visualization Results of PCR-RAPD using OPAH 04 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

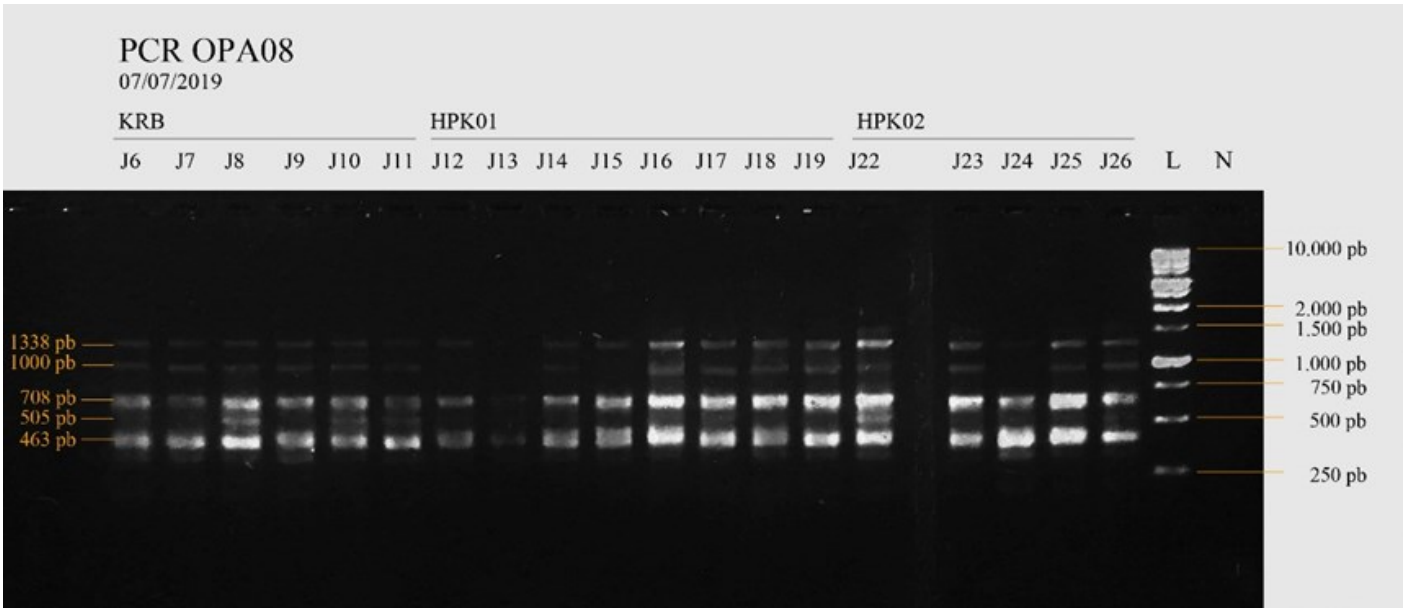

Figure 5. Visualization Results of PCR-RAPD using OPA 08 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

There were 4 loci in the profile RAPD marker using OPAH 04 primer (Figure 4). The lengths of the loci were 918 bp, 533 bp, 510 bp, and 480 bp, respectively. The loci present in the result of PCR-RAPD using OPAH 04 had three conserve alleles in all samples.

There were 5 loci in the electrophoresis result of PCR-RAPD using OPA 08 primer (Figure 5). The lengths of the loci were 1338 bp, 1000 bp, 708 bp, 505 bp, and 463 bp. Loci 707 bp and 463 bp had conserved alleles that were present in all samples. Locus allele of 505 bp was present in samples J8, J10, J11, and J22. Locus allele of 1338 bp was absent in sample J13, while locus allele of 1013 bp was also absent in samples J12, J13, J15, and J24.

Six loci were present in the electrophoresis result of PCR-RAPD using GEN 23 primer (Figure 6). The lengths of the loci were 1077 bp, 1000 bp, 699 bp, 515 bp, 485 bp, 272 bp. Locus 1077 bp was absent in sample J24, while 515 bp was absent in samples J6, J16, J17, J18, and J23. Locus 699 bp was conserved locus present in all samples. Locus 1000 bp was present only in sample J23. Meanwhile, locus 485 bp and 272 bp were present in sample J12, J24, and J25.

Six loci were present in the electrophoresis result of PCR-RAPD using OPAH 02 primer (Figure 7). The lengths of the loci were 1468 bp,

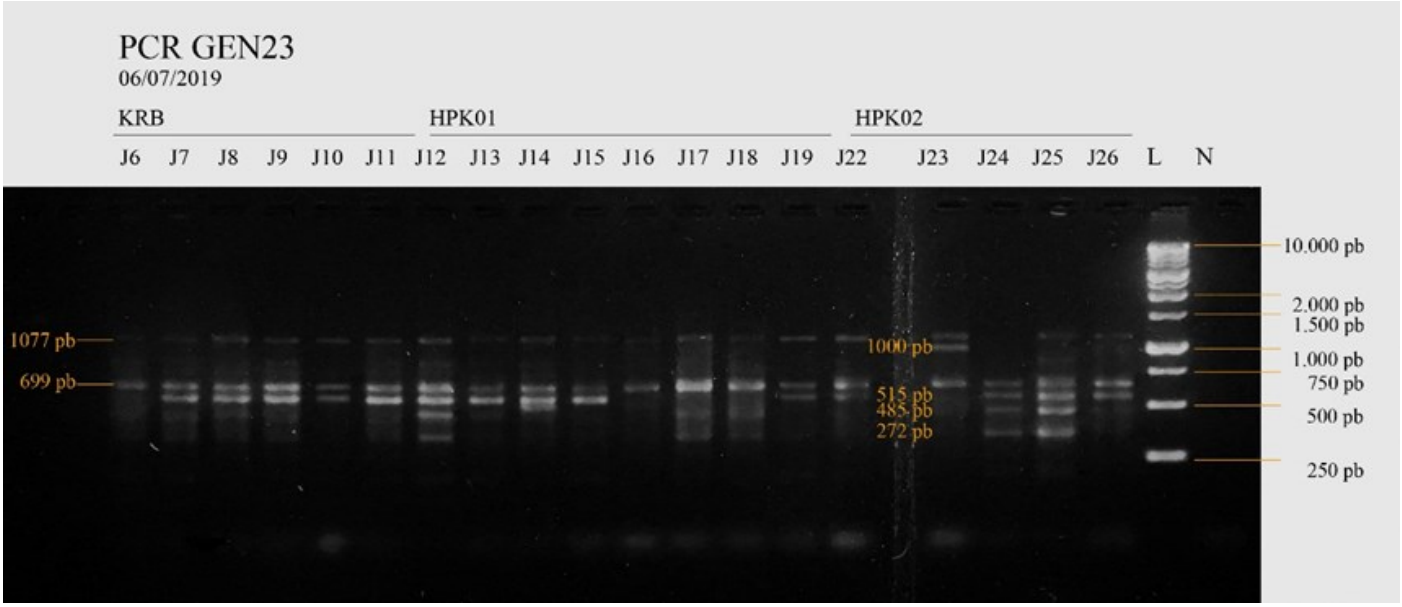

Figure 6. Visualization Results of PCR-RAPD using GEN 23 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

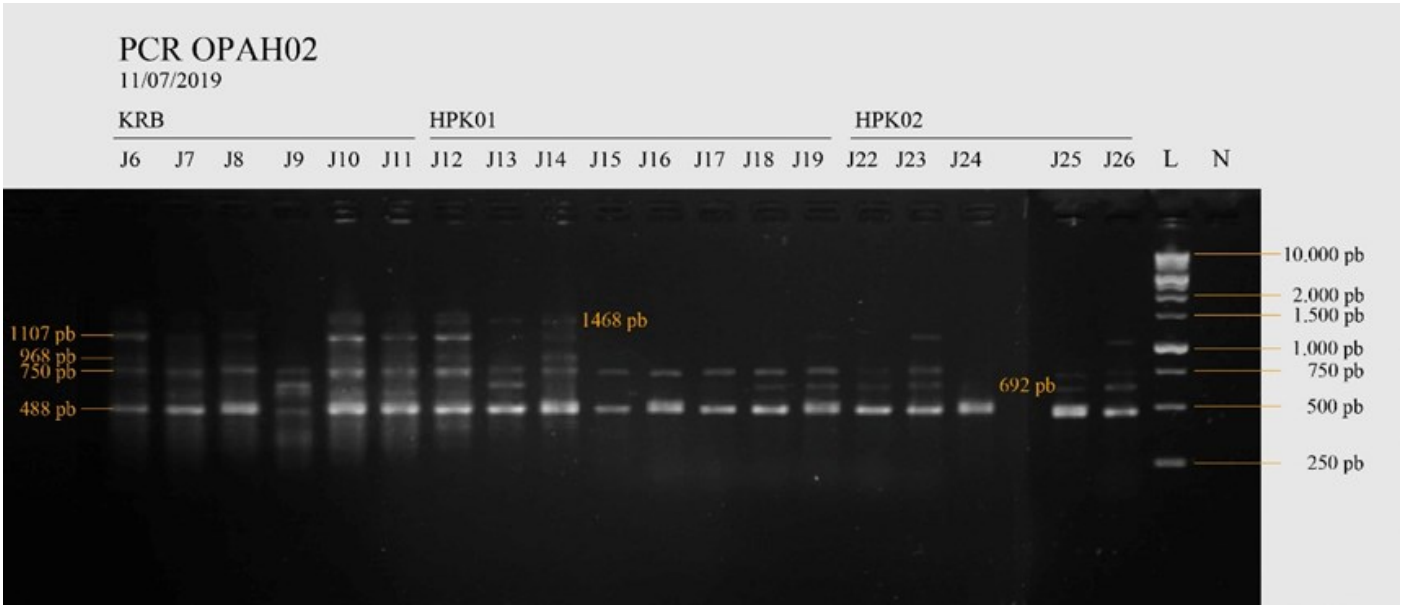

Figure 7. Visualization Results of PCR-RAPD using OPAH 02 Primer on Three *Rhacophorus margaritifer* Populations. L. DNA Ladder; N. Negative Control; KRB. Baturraden Botanical Garden; HPK01. Kalipagu Hiking Trail; HPK02. Baturraden Hiking Trail.

1107 bp, 750 bp, 692 bp, and 488 bp. Locus 1468 bp was present in samples J6, J8, J10, J12, J13, and J14. Locus 1107 bp was present in samples J6, J7, J8, J10, J12, J13, J14, J19, J23, and J26. Locus 968 bp was present in samples J6, J8, J12, and J14. Locus 692 bp was present in samples J9, J13, J18, J19, J22, J23, J25, and J26. Locus 750 bp and 499 bp were present in all samples. There was no band in the negative control of PCR-RAPD using all primers. This meant all bands of *R. margaritifer* DNA produced were specific and usable for analysis.

Amplification results of RAPD markers in part of the samples were smeared although clear bands still present. Smear on the PCR-RAPD result might be caused by Taq polymerase concentration and less optimal DNA template (Williams *et al.*, 1990). Visualised bands also had different contrast aside from smear. The differences of contrast in each sample indicated that extracted DNA genome had different concentrations.

Most of the amplified locus were polymorphic, with the most common allele frequency was more or less than 95%. However, monomorphic locus was also present in amplification result of every primer. This result indicated that those alleles were still [onserved within the DNA genome of *R. margaritifer*.

Table 1. Polymorphism in each primer

Primer	Band length (bp)	Total amplicon produced by individual	Total number of individual	Common allele frequency (%)	Average polymorphism in each primer
OPB 19	1980	2	19	89.47%	
	1514	18	19	94.74%	
	1320	19	19	100.00%	
	1026	17	19	89.47%	
	982	16	19	84.21%	
	928	6	19	68.42%	
	717	19	19	100.00%	91.58%
OPAH 04	918	19	19	100.00%	
	533	19	19	100.00%	
	510	1	19	94.74%	
	480	19	19	100.00%	98.68%
OPA 08	1352	18	19	94.74%	
	1013	15	19	78.95%	
	708	19	19	100.00%	
	505	4	19	78.95%	
	463	19	19	100.00%	90.53%
GEN 23	1077	18	19	94.74%	
	1000	1	19	94.74%	
	699	19	19	100.00%	
	515	14	19	73.68%	
	485	3	19	84.21%	
	272	3	19	84.21%	88.60%
OPAH 02	1468	6	19	68.42%	
	1107	9	19	52.63%	
	968	4	19	78.95%	
	750	18	19	94.74%	
	692	8	19	57.89%	
	488	19	19	100.00%	75.44%

The polymorphic allele was an indication of nitrogen base variation within the DNA genome of *R. margaritifer*. Polymorphic evaluation using OPB 19 and GEN 23 primers managed to point more polymorphic sites within HPK 01 and HPK 02 populations, while OPA 08, OPAH 02, and OPAH 04 primers were able to point polymorphic sites within KRB population. Polymorphic sites of each primer were present in different samples. This could be interpreted that the whole sample had various DNA sequences. Allele might be present and absent because of the differences in complementary sites of RAPD primers. The differences of complementary sites in each sample might be the result of mutation and recombination (Kumar & Gurusubramanian, 2011). Insertion, deletion, or substitution of nitrogen base might cause the primer fails to attach if the original complementary site changed. Mutation also caused new attachment site. Williams *et al.* (1990) stated that the change of one nitrogen base, a single change in primer or DNA template, might affect the primer attachment in DNA genome.

Intra populational analysis using Arlequin *ver.* 3.5 showed high genetic diversity in all populations. Gene diversity in KRB population was 1.0000 ±0.0962 with the average value of locus variation was 0.159524 ±0.105553. Genetic diversity in HPK 01 population was 0.9643 ±0.0772 with the average value of locus variation was 0.165816 ±0.103543. Meanwhile, genetic diversity in HPK 02 population was 1.0000 ±0.1265 with the average value of locus variation was 0.192857 ±0.130697. Although the genetic diversity of KRB population was higher than HPK 01, the average value of locus variation was higher in HPK 01 population than KRB. Sample of HPK 01 population had higher polymorphic sites than sample of KRB population that lead to this result. Meanwhile, the number of polymorphic locus of HPK 02 and HPK 01 were the same, despite higher locus variation in HPK 02. This was caused by the number of gene copy of HPK 01 was higher than haplotype. If genetic diversity value was almost 1 it meant that the population has higher genetic diversity (Nei, 1987). In this case, all three

Table 2. Inter and intra population genetic diversity of *R. margaritifer* in Baturraden

Population	Samples	Haplotype	Loci	Polymorphic Site	Genetic diversity	Locus diversity
KRB	6	6	28	10	1.0000	0.159524
HPK 01	8	7	28	12	0.9643	0.165816
HPK 02	5	5	28	12	1.0000	0.192857

P-value = 0.50244±0.01368

populations of *R. margaritifer* had high genetic diversity that enabled the species to be conserved. A species need to have genetic diversity to adapt with the environmental changes (Nuryanto & Susanto, 2010).

Analysis Molecular of Variance (AMOVA) indicated that there was no genetic difference among populations of *R. margaritifer* in Baturraden (P-value = 0.50244 ±0.01368). This result indicated that the *R. margaritifer* populations in Baturraden were not fragmented. It is could be due to that high gene flow was occurred among *R. margaritifer* populations in Baturraden since lack of prominent barriers among the populations. Short distance (less than 5 km) enabled the migration of individuals among populations and lead high gene flow to occur among populations. Previous studies had shown that geographical distance was significantly correlated with population structure (Silva *et al*., 2007; Telles *et al*., 2006).

CONCLUSION

High genetic diversity had been observed in all populations of *R. margaritifer* in Baturraden, namely 100% in KRB and HPK 02 populations and 96.43% in HPK 01 population. A high locus variation was also observed for all populations with a value of 15.9524% in KRB; 16.5816% in HPK 01; and 19.2857% in HPK 02. There was no genetic difference among populations of *R. margaritifer*.

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