Genetic Variation at Microsatellite Loci in Odorrana hosii (Boulenger, 1891)

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ABSTRACT
The true frog species, Odorrana hosii, found in West Sumatera Indonesia, has high morphological differentiation and also estimated has high genetic variation. A total of 35 O. hosii at a seven location have analyzed using DNA microsatellite markers. Genetic variation of five microsatellite loci provided the highest value of expected heterozygosity (He) for the population in Padang (H_e = 0.618), while the lowest was the population in Merapi (H_e = 0.427). There are genetic differences in moderate levels among populations of O. hosii in West Sumatra (F_ST = 0.108) with inbreeding intrapopulation value (F_IS = 0.559), and high value of gene flow among the populations (Nm = 2.061). This study becomes the first molecular data for establishing effective population management conservation.

Keywords: gene flow; heterozygosity; Odorrana hosii; poisonous rock-frog; Ranidae

INTRODUCTION
The distribution of Odorrana hosii is wide, from Thailand, Malay Peninsula, Sumatra, Java, to Borneo. This species can live in an altitude interval from 300 masl to 1430 masl. The widespread and the altitude interval for survival provide a possibility of morphological and genetic variation to O. hosii (van Kampen, 1923; Inger et al., 2017).

According to a previous study, O. hosii in West Sumatra from different altitudes interval shows a high morphological differentiation and variation to the relative length of chromosomes (Agesi, 2011). Several environmental factors affect differentiation in phenotypes, such as temperature and altitude, genetic factor, or interaction of both factors (Conover & Schultz, 1995; Vences et al., 2002). Therefore, further research is necessary to examine the DNA level among O. hosii in West Sumatra to describe the species morphological variation that also happens genetically. This research aimed to analyze the genetic variation level of the O. hosii population in West Sumatra based on microsatellite markers. DNA Microsatellite markers are used because of these markers have a high polymorphism level. Hence, it is very appropriate to determine the genetic variation level within and between populations (Allendorf et al., 2012). Besides, the analysis of DNA variation using DNA microsatellite can also determine the genetic differentiation of a frog population-based on geographical location with a range of 10 kilometers (Morgan et al., 2008).

MATERIALS AND METHODS
The samples were collected at seven sites in West Sumatra (Alahan Panjang, Padang, Pasaman, Palupuah, Payakumbuh, Gunung Merapi, and Sijunjung). Those locations have been chosen based on altitude and distance shown in Figure 1 and Table 1.

DNA isolation. Analysis of genetic variation used DNA microsatellite markers (Funk et al., 2005; Arruda et al., 2017). DNA isolated from the liver tissue of five individuals from each population using QIAGEN kit protocol DNAeasy® Blood and Tissue Kit. PCR reaction was performed using PROMEGA PCR Core kit by mixing 2 μl isolate DNA, 12 μl GoTaq Green Master Mix, 1 μl forward primer, 1 μl reverse primer, and sterile water until the total volume is 25 μl. All loci have an optimal temperature. Information of nine microsatellite loci showed in Table 2. Amplification of DNA was programmed with an initial denaturation 95°C for 2 min, 33 cycles of denaturation: 95°C for 30 s, annealing: at optimal temperature 30 s, extension: 72°C for 30 s, and final extension 72°C for 5 min. The product was detected by electrophoresis in
agarose gel (1.2% of DNA isolation and 2% of the PCR product).

Figure 1. Research sites of *Odorrana hosii* in West Sumatra

**Data analysis.** Quantification of the results analyzed using the GENEPOP version 4.0 to determine the genetic variation within and between populations. The parameters used to determine the genetic variation within a population were allele frequencies, the percentage of polymorphic loci \( (P_p) \), the average number of alleles per locus \( (N_a) \), and expected heterozygosity \( (H_e) \). Genetic variation between populations can be seen from the F-statistic, genetic distance and similarity values dendrogram based on Nei’s genetic distance matrix (1978) and the value of gene flow \( (Nm) \) (Raymond & Rousset, 1995).

Table 1. Geographic position and attitude of research sites of *O. hosii*

| No. | Location                        | District          | Altitude (m a.s.l) | Geographic Position       |
|-----|---------------------------------|-------------------|--------------------|---------------------------|
| 1   | Alahan Panjang                  | Solok             | 1200-1400          | 1°08’ SL and 100°07’ EL   |
| 2   | Cagar Alam Lembah Harau-Payakumbuh | 50 Kota          | 500-600            | 0°04’ SL and 100°39’ EL   |
| 3   | Cagar Alam Malampah-Pasaman Barat | Pasaman Barat    | 456-763            | 0°11’ SL and 100°04’ EL   |
| 4   | Hutan Pendidikan Penelitian Biologi (HPPB) Unand Padang | Padang        | 260–300            | 0°21’ SL and 100°46’ EL   |
| 5   | Gunung Merapi                   | Agam              | 900-1500           | 0°22’ SL and 100°28’ EL   |
| 6   | Cagar Alam Pangean I-Sijunjung  | Sawahlunto Sijunjung | 300-600         | 0°90’ SL and 101°50’ EL   |
| 7   | Cagar Alam Batang Palupuah-Agam | Agam              | 700-1000           | 0°14’ SL and 100°21’ EL   |
RESULT AND DISCUSSION

Amplification results of nine loci showed that only five loci produce the amplicon (Rnh-3, Rnh-9, Rnh-10, Rnh-12, and Rnh-13). The optimal temperature has been searched previously for the nine loci with different annealing temperatures. The annealing temperature for the five loci were modified from Gong et al. (2013) shown in Table 2.

Table 2. Nine primer pairs of microsatellite markers produces amplicons and annealing temperature

| Locus ID | T_a (°C) | Repeat Motif | Primer Sequences (5'→3') | Amplicon |
|----------|----------|--------------|---------------------------|----------|
| Rnh-1    | -        | (TGC)7       | F: TGAAGTATTCAGGTACAACAGGT | X        |
|          |          |              | R: GGGCCAAAAGAGAGGGT       |          |
| Rnh-2    | -        | (TGC)4TGTT(GTT)3T | F: GCTTGGGCTATAATCAAACA | X        |
|          |          | (TGC)6       | R: GCCCTGGCCGACTACACG      |          |
| Rnh-3    | 52       | (AAC)7       | F: CGGAAGGCAGTGAGGAGCA     | V        |
|          |          |              | R: ATGGACATGCGGTGGGTAGG    | X        |
| Rnh-4    | -        | (GA)5        | F: CGTTACTATGGGGGATA       | X        |
|          |          |              | R: GCCCTGAAGGGTGTTGCT      |          |
| Rnh-6    | -        | (AAC)5       | F: TCTCGGGAGGAAAGCATAAGG  | V        |
|          |          |              | R: AAGGAGCCTTGGACTATGTTAAC |          |
| Rnh-9    | 59.5     | (GCA)7       | F: GCACAGTTAGCGAGATGGA     | V        |
|          |          |              | R: CTCACCTAGCTGGTTGATAT    |          |
| Rnh-10   | 61       | (GCT)6       | F: AGTGCAACATCAACTTGGGTG  | V        |
|          |          |              | R: GCAGCAGTGGCTTGGGA       |          |
| Rnh-12   | 58.5     | (AC)20       | F: ATGTTATCGAGCCAGAG      | V        |
|          |          |              | R: GGTCAGCGAGGTAA         | X        |
| Rnh-13   | 55.3     | (GCA)5       | F: GATACGGGAGGCAACAGC     | V        |
|          |          |              | R: TCCACAGCCCAGCACTC      |          |

Notes: T_a = annealing temperature; V = amplicon produces; X = not amplicon produces

The number of alleles at each locus ranged from five to seven, with as much as 27 alleles and an average of 5.4 alleles per locus. All alleles microsatellite showed a high polymorphism with the percentage of polymorphic loci (P_p) ranged from 75 (Rnh-13) to 100% (Rnh-9). Analysis of genetic variation showed that population of Padang have a high genetic variation with 17 allele per locus, 0.618 expected heterozygosity (H_e), Shannon Index of 1.00, and 100% of polymorphic loci (P_p), and Merapi have a lower (N_a = 11; H_e = 0.427; I = 0.616; P_p = 80%)(Table 3).

Table 3. Analysis of the genetic variation of O. hosii based on microsatellite DNA in seven populations

| No. | Population | N_a | H_e | I   |
|-----|------------|-----|-----|-----|
| 1   | Padang     | 17  | 0.618 | 1.000 |
| 2   | Alahan Panjang | 13  | 0.529 | 0.767 |
| 3   | Malampah   | 13  | 0.533 | 0.783 |
| 4   | Harau      | 11  | 0.444 | 0.619 |
| 5   | Palupuh    | 13  | 0.596 | 0.846 |
| 6   | Sijunjung  | 12  | 0.520 | 0.733 |
| 7   | Merapi     | 11  | 0.427 | 0.616 |
| Mean|             | 12.86 | 0.524 | 0.766 |

Notes: N_a = the number of alleles per locus; H_e = expected heterozygosity; I = the average of Shannon index

The high value of H_e in Padang because of large population sizes. A large number of O. hosii populations were based on criteria of high-level occurrence of O. hosii whilst collecting samples. Environmental conditions supported the local adaptation of populations (Arens et al., 2006; Alberto et al., 2013). It has known that Padang was likely to become the habitat of O. hosii. According to Iskandar (1998), O. hosii was likely to live on a clear and strong water stream and a river streamline in the primary forest. A large population will reduce
the chances of inbreeding and increase random mating so that heterozygosity will also be increased (Cervantes et al., 2011; Frankham, 2015; Hedrick & Garcia-Dorado, 2016).

The lowest \( H_e \) value was found in the Merapi population. It can expect these values a small number of population, which will increase the occurrence of inbreeding (Frankham et al., 2002). Inbreeding associated with the loss of genetic variation, drift and less heterozygous levels related to the loss of some alleles and low levels of polymorphism. As a result, it would increase inbreeding depression (Arens et al., 2006; Taylor et al., 2010; Jamieson, 2011).

Inbreeding depression is mating between individuals from the same parental offspring, which produce offspring with lower live quality level (Arens et al., 2006). Besides, Allendorf et al. (2012) also stated that a low level of heterozygosity could reduce viability because most of the population recessive alleles were lethal. Lethal recessive alleles, predominantly covered by dominant alleles in a heterozygote locus, have a big opportunity to express in a few populations. The extinction will probably led faster in a small number of frog populations (Rog et al., 2013; Soto-Azat et al., 2013). The categorization of a population can be looked at from its frequency of occurrence. The frequency of occurrence of \textit{O. hosii} from Merapi is relatively lower than another population.

Table 4. The value of F-statistik (\( F_{ST}, F_{IS}, F_{IT} \)) and gene flow (\( Nm \))

| Samples number | \( F_{ST} \) | \( F_{IS} \) | \( F_{IT} \) | \( Nm \) |
|----------------|-------------|-------------|-------------|--------|
| 35             | 0.108       | -0.559      | -0.3904     | 2.061  |

Notes: \( F_{ST} \) = coefficient of genetic differentiation; \( F_{IT} \) = the total inbreeding value; \( F_{IS} \) = inbreeding intra population; \( Nm \) = gene flow

\( F_{ST} \) value (Table 4) can determine the level of genetic differentiation between the \textit{O. hosii} population in West Sumatra. According to Wright (1938), there are four categories of \( F_{ST} \) values that ranged from 0 to 0.05; 0.05-0.15; 0.15-0.25; and >0.25. Each showed a level of genetic differentiation, which indicated low, moderate, high, and very high genetic differentiation. Based on the statements above, we can conclude that \textit{O. hosii} between populations in West Sumatra has genetic differentiation value that shows moderate genetic differentiation (\( F_{ST} = 0.108 \)). From the \( F_{ST} \) value, we can determine that 10.82% of total genetic variation is among populations, and 89.18% is within the population.

The \( F_{IT} \) value that is less than 0 indicated that inbreeding in a population is low and random mating still occurs, so the probability for closely related species cross is low (Charlesworth & Willis, 2009). It is also supported by the value \( Nm \) among populations (2.061; \( Nm \leq 0.5 \)). Based on that value, we know the migration of individuals among the population of \textit{O. hosii} in West Sumatra is high (Slatkin, 1981). According to the obtained value, it can be concluded that \textit{O. hosii} among populations in West Sumatra does not have genetic differentiation significantly based on microsatellite DNA.
The pattern of *O. hosii* grouping among populations is presented in the dendrogram (Figure 2). Based on the UPGMA cluster analysis, *O. hosii* grouping in West Sumatra is divided into two major groups and provides a unique grouping pattern. *O. hosii* populations form groups following Bukit Barisan highlands (Padang and Malampah populations create a group on the western side of Bukit Barisan highlands; Alahan Panjang, Sijunjung, Harau, Merapi, and Palupuh populations create a group on the eastern side of Bukit Barisan), as well as the existence of rivers that facilitate the migration of individuals among populations. *O. hosii* grouping based on UPGMA cluster analysis is supported by genetic distance value among populations (Table 5).

| No. | Population | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|-----|------------|-----|-----|-----|-----|-----|-----|-----|
| 1   | Padang     | 0.000 |     |     |     |     |     |     |
| 2   | Alahan Panjang | 0.070 | 0.000 |     |     |     |     |     |
| 3   | Malampah   | 0.080 | 0.126 | 0.000 |     |     |     |     |
| 4   | Harau      | 0.094 | 0.014 | 0.134 | 0.000 |     |     |     |
| 5   | Palupuh    | 0.144 | 0.027 | 0.227 | 0.017 | 0.000 |     |     |
| 6   | Sijunjung  | 0.127 | 0.002 | 0.240 | 0.038 | 0.009 | 0.000 |     |
| 7   | Merapi     | 0.118 | 0.025 | 0.291 | 0.009 | 0.007 | 0.003 | 0.000 |

Table 5 presents that several populations are showing the great value of genetic distance differences among the population. It is caused by factors such as geographical isolation in the form of physical barriers, environmental factor differences, and reproduction. One of those factors will affect genetic structure formation (Arioli et al., 2010; Fouquet et al., 2012; Twomey et al., 2016). It can be concluded that geographical isolation factor, which is Bukit Barisan highlands locating from northern to the southern side of Sumatra that serves as a physical barrier separating among populations, has affected genetic structure formation of *O. hosii*.

**CONCLUSION**

The highest genetic variation of *O. hosii* is from the population in Padang \( (N_a = 17, H_e = 0.618, I = 1.000) \), while the lowest genetic variation is from the population in Merapi \( (N_a = 11, H_e = 0.427, I = 0.616) \). The existence of Bukit Barisan Mountain potentially becomes a barrier as well as the classification of *O. hosii* in West Sumatra. The analysis of the population's genetic structure has shown a moderate genetic variation \( (F_{ST} = 0.108) \).

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