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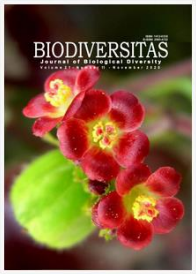

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RAPD based molecular analysis genetic diversity

by Masud Abd

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RAPD based molecular analysis genetic diversity of *Ornithoptera croesus* found in Bacan Island, Indonesia

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Abstract. *Mas'ud A, Corebima AD, Amin M, Rohman F. 2018. RAPD based molecular analysis genetic diversity of* Ornithoptera croesus *found in Bacan Island, Indonesia. Biodiversitas 19: 1273-1279.* The endemic butterfly commonly known as birdwing butterfly found in Bacan island (*Ornithoptera croesus)* was first discovered by Wallace in 1859. The *O. croesus* hotspot is a nature preserve located at various altitudes around Sibela mountain. The *O. croesus* belongs to macrolepidoptera which is famous for their variety of body colors and typical morphology identified in female and male found at various altitude places. This study aimed to examine the genetic diversity of *O. croesus* at some different altitude spots in Sibela mountain using PCR-RAPD molecular analysis. The result of the DNA amplification showed high polymorphism in *O. croesus* (84,81). The clustering pattern indicated that *O. croesus* ♂ 400 meters above sea level (m asl.) and *O. croesus* ♀ 400 m asl. had the highest level of genetic similarity, while the lowest level of genetic similarity was observed in *O. croesus* ♀ 800 m asl. and *O. croesus* ♀ 20 m asl. On the basis of these findings, clustering pattern showed that the highest level similarity could also be found in species that live in the same altitude.

Keywords: Altitudinal, endemic, *Ornithoptera croesus*, similarity

INTRODUCTION

Birdwing butterfly (*Ornithoptera croesus*) is a macrolepidopteran species endemic in Bacan island (Peggie et al. 2005) was first discovered by Wallace in 1859. Morphological variation found in *O. croesus* includes the wing color, wingspan, leg length and body size (Wallace 1869; Collins and Morris 1985; Peggie 2011). Female and male *O. croesus* can be differentiated by its wing color pattern and body size. A number of studies have identified the morphological variation in birdwing butterflies such as Kondo et al. (2003) in *Trogonoptera* spp, *Troides* spp., and *Ornithoptera* spp.; Sullivan and Miller (2007) in macrolepidoptera; Hebel (2010) in butterflies found at different altitude ranges; Suwarno (2010) in population dynamic Swallowtail butterfly in dry and wet season; Makhzuni et al. (2013) Variations Morphometry of butterflies *Papilio polytes* on various plateau and low in West Sumatra; and Despland (2014) in Atacama plateau butterflies. Harmonis and Saud (2017) state that butterfly communities were affected by degradation habitat, while fragmentation habitat did not influence the butterfly communities.

Morphological variation in *O. croesus* has also been found on the hotspots located at some different gradient places. Some research that attempted to reveal intraspecific variation in butterflies and insects based on their habitat and altitudes are Sreekumar and Balakreesnan (2001) in butterflies in India; Hodkinson (2005) in insects found in diverse altitudes; Botes et al. (2005) in Northen ants; Brehm et al. (2007) in night butterfly found in different altitude places; Lamkind et al. (2011) in Diptera; Rotrigers (2008) in hymenoptera; Zarikian (2017) altitudinal distribution of Papilionoidea (Lepidoptera) in Mount Aragats, Armenia.

Mount Sibela is known geographically as the highest mountain in North Maluku. It is also known as the ecological niche of *O. croesus* butterfly (Wallace 1869; Collins and Morris 1985). The *O. croesus* butterflies and other species belong to family Papilionidae can be discovered in the mountain areas at different altitudes ranging from 20 meters above sea level (m asl.) to 800 m asl. Mount Sibela is also a habitat for Mussaenda and Ashoka that provide nutrients for the *O. croesus* butterfly (Mas'ud 2016).

There is no availability of any updated information ~~on~~ regarding morphological and molecular genetic diversity of *O. croesus* butterfly. Thus, this RAPD based study would attempt to fill this gap by examining the genetic diversity (intraspecies) of the endemic butterfly of Bacan island (*O. croesus*) at various altitude places in Mount Sibela. This present study was the first attempt to preserve the endemic butterfly of North Maluku conducted on the intraspecies diversity of *O. croesus* based on molecular character. This study was also done as an effort to preserve the endemic butterfly of North Maluku, Indonesia.

MATERIALS AND METHODS

Specimen collection

Male and female adult *O. croesus* butterflies were caught and collected by using an insect net from four locations in Mount Sibela conservation area (20 m asl., 200 m asl., 400 m asl., and 800 m asl.) (Figure 1). Purposive sampling technique was used to select the samples. The samples consisted of four male and four female adult butterflies (Figure 2). These fresh samples were washed by using 70% alcohol and preserved. Dried specimens were taken into Life Science Central Laboratory of Brawijaya University, Malang, Indonesia for molecular analysis

Isolation of total DNA

DNA was isolated from the legs of the specimens (80 mg). DNA isolation followed the Nucleospin Genomic DNA protocols using a miniprep kit (Macherey-Nagel). This kit employed a column purification technique to extract total DNA from an animal tissue. The cell undergoes lysis by grinding it in a T1 lysis buffer (180 µL) and adding it with 25 µL pro-K. It was then incubated inside a thermomixer at 56°C, shaken at 500 rpm for 3 hours, and centrifuged at 11000*xg*, 25°C for 5 minutes. The supernatant was transferred into a new tube and added with 200 µL buffer B3 and mixed gently until it was homogeneous. It was incubated in a thermomixer at 70°C for 30 minutes, shaken at 400 rpm, added with 210 µL non vortex absolute ethanol, and centrifuged at 11000*xg* for 1 minute (25°C). The column was replaced, added with 500 µL buffer BW, and centrifuged at 11000*xg* for 1 minute (25°C). The column was substituted, added with 600 µL buffer BS, and centrifuged at 11000*xg* for 1 minute (25°C). The dry silica membrane was centrifuged at 11000*xg* for 1 minute (25°C). The tube was transferred into a new 1,5 mL tube, added with 25 µL hot buffer BE solution (70°C), incubated for 5 minutes at the room temperature, and centrifuged at 11000*xg* for 3 minutes (25°C). The solution was added with 25 µL hot buffer BE, incubated for 5 minutes at the room temperature and centrifuged at 11000xg 25°C to obtain final DNA.

Polymerase Chain Reaction (PCR)

The PCR analysis followed the protocols provided by PCR *Master Mix* (Intron) Kit. The PCR (total volume = 10 µL) was composed of primer 10 pmol/µL (1 µL); ddH_2O (2.75 µL), PCR Mix (5 µL), BSA 10 mg/ML (0.25 µL), and DNA (1 µL). Primers used were OPA 1-OPA 20 (Table 1). The PCR temperature control was described as follows. Initial denaturation was performed at 92ºC for 4 minutes, and continued in 45 cycles (denaturation: 92ºC, 2 minutes; primers attachment: 36ºC, 60 seconds; and DNA elongation: 72ºC, 120 seconds and post extension 72ºC, 10 minutes). DNA bands were electrophoresed using agarose gel 1 % and visualized using a UV-transilluminator.

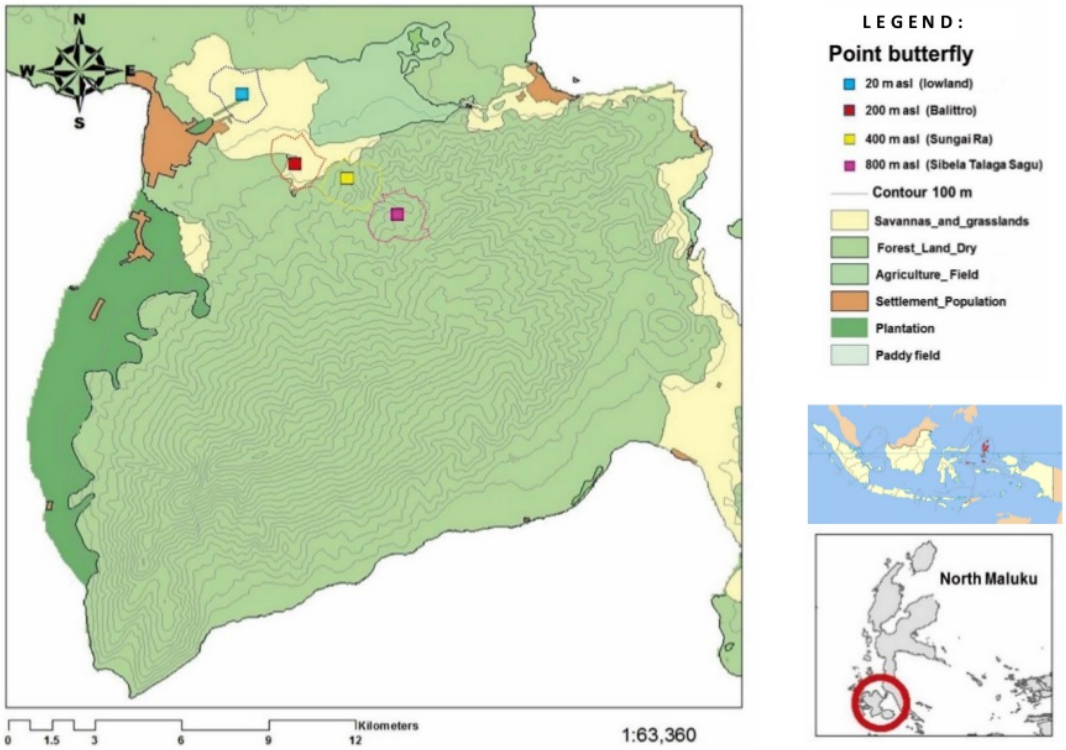

Figure 1. Locations of *O. croesus* sample collection in Mount Sibela conservation area, Ternate, North Maluku, Indonesia

Data analysis

Data were analyzed based on the existence of DNA bands. Zero (0) means that no DNA band found while one (1) signified the existence of a DNA band. Cluster analysis was performed using the UPGMA (Unweight Pair Group Method with Arithmetic Mean) technique, Multivariate Statistical Package (MVSP) program version 3.22 (Kovach: 2007)

RESULTS AND DISCUSSION

Research data was reported based on the existence of *O. croesus* DNA bands in eight individuals (4 males and 4 females) collected from various altitudinal locations in Mount Sibela conservation area (Table 1).

In total, there were 180 bands were obtained based on the existence of DNA band pattern shown in the pictures and identified with the following criteria: 158 were polymorphic and 22 were monomorphic. The average percentage of polymorphism in primers OPA 1-20 was 84,81% (Table 1). Based on polymorphic values it is known that there is high genetic diversity signifies there are many variations in phenotype and genotype characteristics in the *O croesus* butterflies. Intraspecies variations were found in the butterfly *O croesus* After that, an analysis of matrix similarity was conducted very shortly based on the appearance of DNA bands (DNA profile) with scoring 1 (present) and 0 (absent) (Figure 2).

Based on the results of the RAPD visualization (Figure 3), matrix analysis could be performed (Table 2). The highest value of matrix similarity (0,839) (Table 2) was observed in *O. croesus* ♂ 400 m asl. and *O. croesus* ♀ 400 m asl. The results of the RAPD analysis indicated that there were a lot of similarities that could be found between *O. croesus* ♂ and *O. croesus* ♀ collected at 400 m asl. Meanwhile, the lowest level of similarity (0,642) was identified in *O. croesus* ♀ 800 m asl. and *O. croesus* ♀ 20 m asl. Dendrogram generated from the average value of matrix similarity in 20 OPA primers is presented in Figure 4.

The result of UPGMA (dendrogram) analysis shows that the similarity value (0,52) is the root that makes up 4 main clusters: main cluster I consisted of *O. croesus* ♀ 800 m asl. and *O. croesus* ♀ 20 m asl., main cluster II consisted of *O. croesus* ♀ 200 m asl., main cluster III consisted of *O. croesus* ♂ 800 m asl., *O. croesus* ♀ 400 m asl., *O. croesus* ♂ 400 m asl., and main cluster IV consisted of *O. croesus* ♂ 200 m asl. and *O. croesus* ♂ 20 m asl. The highest level of similarity (0,84) was found between *O. croesus* ♀ 400 m asl. and *O. croesus* ♂ 400 m asl. This finding suggests that butterflies from the highlands tend to have higher levels of genetic similarity, whereas those from the lowlands tend to exhibit random patterns. It is suspected that the direction of the spread pattern of this butterfly extends from the plateau to the lowlands.

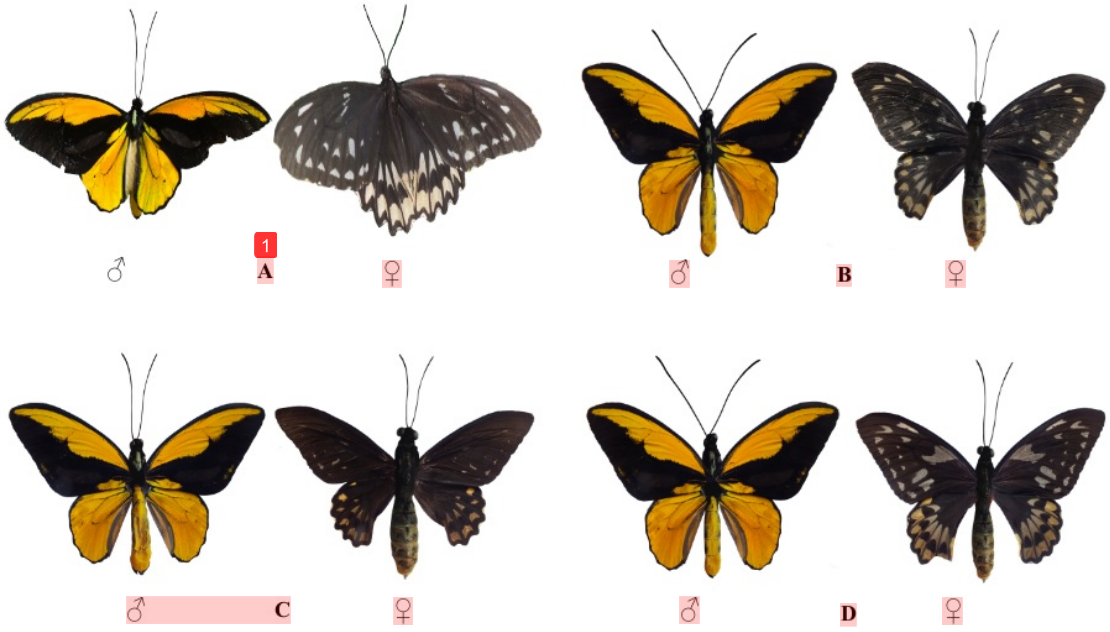

Figure 2. The endemic butterflies of Bacan island (*O. croesus)* collected from four locations. A = 20 m asl., B = 200 m asl., C = 400 m asl. and D = 800 m asl.

Table 1. RAPD primers, sequence and polymorphism (%) of *O. croesus* analyzed based on the existence of DNA-RAPD bands pattern using the UPGMA method

Primer	Seq 5 to 3	Seq 5 to 3 bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)
OPA-1	CAG GCC CTT C	07	06	01	85.71
OPA-2	TGC CGA GCT G	03	02	01	66.66
OPA-3	AGT CAG CCA C	11	11	00	100
OPA-4	AAT CGG GCT G	07	04	03	57.14
OPA-5	AGG GGT CTT G	13	11	02	84.61
OPA-6	GGT CCC TGA C	05	05	00	100
OPA-7	GAA ACG GGT G	10	09	01	90
OPA-8	GTG ACG TAG G	09	08	01	88.88
OPA-9	GGG TAA CGC C	09	09	00	100
OPA-10	GTG ATC GCA G	08	06	02	75
OPA-11	CAA TCG CCG T	17	17	00	100
OPA-12	TCG GCG ATA G	08	08	00	100
OPA-13	CAG CAC CCA C	08	05	03	62.5
OPA-14	TCT GTC CTG G	09	09	00	100
OPA-15	TTC CGA ACC C	09	09	00	100
OPA-16	AGC CAG CGA A	07	06	01	85.71
OPA-17	GAC CGC TTG T	08	06	02	75
OPA-18	AGG TGA CCG T	06	02	04	33.33
OPA-19	CAA ACG TCG G	14	14	00	100
OPA-20	GTT GCG ATC C	12	11	01	91.66
Total		**180**	**158**	**22**	**84.81**

Table 2. The *O. croesus* matrix similarity based on the existence of DNA-RAPD analyzed using the UPGMA method

	O. ♂ **20 m asl.**	*O. c* ♀ **20 m asl.**	*O. c* (♂) **200 m asl.**	*O. c* (♀) **200 m asl.**	*O. c* (♂) **400 m asl.**	*O. c* (♀) **400 m asl.**	*O. c* (♂) **800 m asl.**	*O. c* (♀) **800 m asl.**
O. croesus ♂ 20 m asl.	1							
O. croesus ♀ 20 m asl.	0.650	1						
O. croesus ♂ 200 m asl.	0.763	0.600	1					
O. croesus ♀ 200 m asl.	0.644	0.650	0.761	1				
O. croesus ♂ 400 m asl.	0.689	0.672	0.739	0.678	1			
O. croesus ♀ 400 m asl.	0.733	0.606	0.772	0.689	0.839	1		
O. croesus ♂ 800 m asl.	0.639	0.656	0.744	0.639	0.761	0.833	1	
O. croesus ♀ 800 m asl.	0.650	0.642	0.659	0.653	0.653	0.678	0.656	1

The results of the present study also revealed the similar patterns of *O. croesus* butterflies based on gender. Main cluster I, for example, was dominated by female butterflies collected from the far away hotspots at 20 m asl. and 800 m asl. Meanwhile, main cluster IV consisted of male butterflies coming from locations at 20 m asl. and 200 m asl. This clustering pattern based on gender was reported to have lower level of similarity compared to the clustering pattern based on the butterflies hotspots. The results of the analysis on molecular character of *O. croesus* showed high similarity between female and male butterflies collected at 400 m asl. (Table 2). This finding has corroborated with those reported about studies of the influence of altitude on insect body size (Hawskin & Devries, 1996); altitude influences the genetic diversity and abundance of butterflies (Brehm et al, 2003; Brehm et al, 2007); the influence of altitude on the structure of butterfly community in India (Prakash et al., 2007); and the relationship between altitude and the diversity of flies (Lamkin et al 2011), by Hawskin and Devries (1996); Brehm et al. (2003); Brehm et al. (2007); Prakash et al. (2007); and Lamkin et al. (2011).

The genetic diversity of butterflies can be analyzed based on their morphological character (Makhzuni et al. 2013) or molecular data (Vijay et al. 2010; Tiple et al. 2010; Zothansangi et al. 2011). PCR-RAPD molecular marker can be used to examine DNA polymorphism, gene flow between populations, evaluation of genetic population structure, genetic, and phylogenetic determinations (Zulfahmi, 2013). There are two factors that might influence the genetic diversity of *O. croesus*: internal factors and external factor. The internal factors include genetic variation based on the polymorphic value and RAPD analysis and gene recombination. Meanwhile, the external factors consist of the environment and the habitat carrying capacity including the hostplant provision. Temperature, rainfall, and altitudes are some environmental factors that can affect the *O. croesus* genetic diversity. The results of the analysis of habitat condition related to the hostplant provision and the number of *O. croesus* individuals in four research locations are summarized in Table 3.

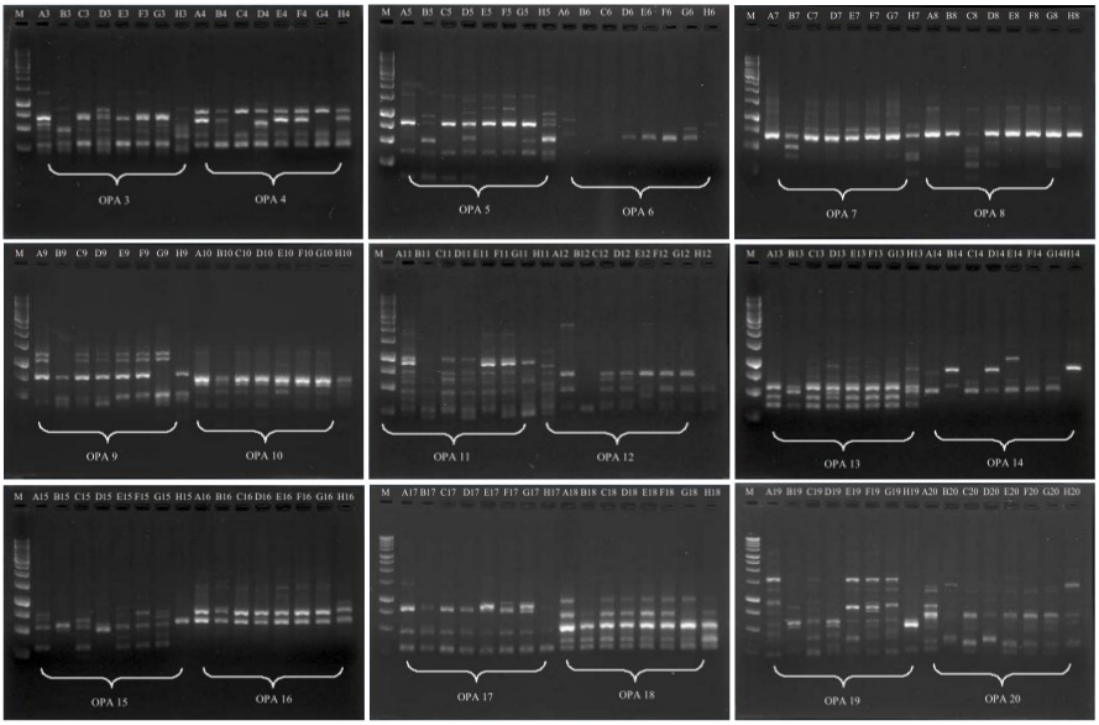

Figure 3. Visualization of RAPD bands in 8 *O. croesus* individuals with primers OPA 1-20

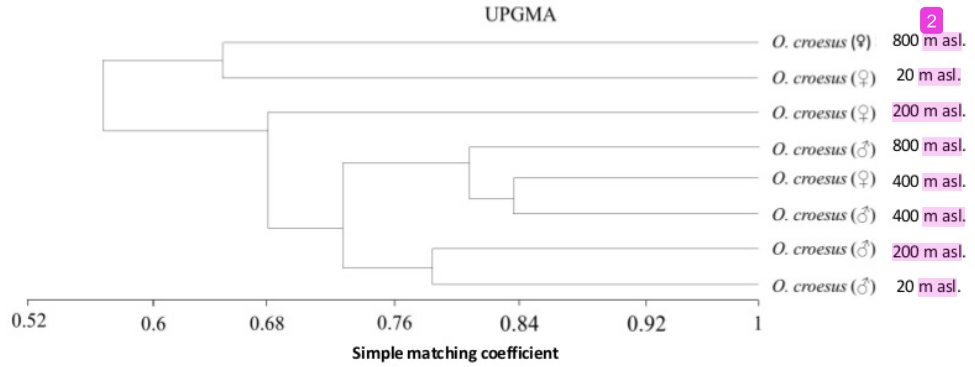

Figure 4. The dendrogram of eight *O. croesus* individuals based on the DNA-RAPD pattern analyzed using the UPGMA method

Table 3. Habitat condition and the number of *O. croesus* individuals in four research locations

Location	Altitude	Dominant hostplant	Number of plant per m^2	Number of *O. croesus* individual per m^2
Settlement	20 m asl.	Mussaenda	33 trees	16
		Ashoka	13 trees	8
Plantation	200 m asl.	Musaenda	21 trees	12
		Ashoka	11 trees	6
Production Forest	400 m asl.	Mussaenda	15 trees	9
		Ashoka	9 trees	4
		Gusale	15 trees	4
Limited Conversion Forest	800 m asl.	Mussaenda	9 trees	4
		Gusale	58 trees	4

Mussaenda and Ashoka trees provide nutrients for *O. croesus* butterflies. These decorative plants can be found in low altitude areas around Mount Sibela. Besides Mussaenda and Ashoka, the *O. croesus* butterflies also feed on Gusale tree that can be found at around 800 m asl. that's why they mostly live in the lowlands due to the provision of food. This result is inconsistent with that of Mas'ud and Corebima (2016) who suggested that there was a relationship between the number of Mussaenda and Ashoka plants and the population density of *O. croesus* in Bacan island. Moreover, Collinge et al. (2003) also state that thick meadows are the perfect habitat for various butterflies. In addition, findings by Joshi and Sharma (2009) indicate a correlation between the complexity of a structural habitat, vegetation forms, and butterflies diversity. High vegetation diversity will result in high butterflies diversity (Van Vu, 2011). The components of carrying capacities such as the provision of shelter, water, mineral, food, temperature, and moisture (Ruslan, 2009) also have an effect on the diversity of butterflies. Needless to say, the number of *O. croesus* individuals and the provision of hostplant are also affected by the geographical conditions including extreme altitudes and natural landscapes (Sullivan and Miller,2007).

The genetic diversity (intraspecies) of *O. croesus* in Bacan island is high (84,81%) which suggests that *O. croesus* has genetic variation in one species. High genetic diversity is one of the sources of biodiversity that possesses complimentary vitality. Cluster between female *O. croesus* at 20 m asl. and 800 m asl. indicates high intraspecies diversity. It can be assumed that *O. croesus* butterflies migrated from high lands (800 m asl.) to low lands (20 m asl.) to find Mussaenda and Ashoka trees as their food (Table 3). The existence of butterflies in their ecological niche highly depends on the environment carrying capacity that is related to the provision of host plant and food plant. Host plant is a plant that provides nutrients for butterfly larvae while food plant is a plant that is feed on by the adult butterfly (Sodiq 2009; Shalihah et al. 2012).

In conclusion, the results of the PCR-RAPD analysis suggested that the endemic butterflies of Bacan island *O. croesus* had high diversity with high polymorphism (84,81%). The highest level of similarity was observed in male and female *O. croesus* butterflies which come from an altitude spot of 400 m asl. The clustering pattern showed that the highest level similarity could also be found in species that live in the same altitude.

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6	issuu.com Internet Source	<%1
7	www.archive.org Internet Source	<%1
8	citeseerx.ist.psu.edu Internet Source	<%1
9	d-nb.info Internet Source	<%1

10	bioone.org Internet Source	<%1
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