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Research Article
Genetic Variability of *Ornithoptera croesus toeantei* Endemic Butterfly in Morotai Island, Based on Morphology and Molecular

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Abstract

Background and Objective: *Ornithoptera croesus toeantei* is an endemic butterfly in the island of Morotai north Maluku discovered by Parrot and Schmid at 1894. The existence of this butterfly as an endemic species has not been published yet. The objective of this study is to analyze the genetic variability of *O.c. toeantei* based on the morphological characters and molecular PCR-RAPD in several level of altitude of hotspots on the Morotai Island. **Materials and Methods:** This research was conducted using the morphometric description method and the RAPD-PCR method. The data were analyzed descriptively qualitatively in the UPGMA cluster pattern using the MPSV program version 3.22. **Results:** The result of the study informs that in general an endemic of *O.c. toeantei* has a high genetic diversity on intraspecies level. The value of 0.56 on the morphometric dendrogram and 0.56 on the molecular dendrogram RAPD showed that the similarity between clusters on morphological characters is in line with the character of molecular-RAPD. **Conclusion:** It can be concluded that there is a similarity on the clustering pattern between UPGMA analysis at morphometric and molecular-RAPD character of *O.c. toeantei* which showed that the level of the altitude gives some influences to the genetic diversity.

Key words: Morphometry, molecular-RAPD, *O.c. toeantei*, endemic, Morotai Island

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ornithoptera spp. is one of the genus of birdwing butterfly. The attractiveness of this butterflies lies on its golden yellow color with unique pattern[1,2]. The caput, torax, antenna, proboscis in both male and female generally have a black and dark brown colors. However, the male usually can be identified from the black color with golden yellow strip encircle and lined up in the middle, while its abdomen has a yellow color. The female, on the other hand, generally has dark brown with several golden white or golden yellow spots, brownish white at the abdomen and yellow at the bottom[1-3].

Ornithoptera croesus toeantei is categorized into the macrolepidoptera birdwing group, an endemic butterfly on the Morotai Island North Maluku and firstly discovered by Collins and Morris[2], Peggie[3]. Morotai Island itself has a total area of 4,301.53 km^2, with 2,314.90 km^2 of land area and 4 mile of the sea covering of 1,970.93 km^2 area with the altitude of 0-1000 masl[4]. Geographically, Morotai Island is categorised into one of what its known in Indonesia as 3T (lagging, leading, outermost) frontier regions or in other words the region that still underdeveloped[4]. This Island is separate from the mainland of Halmahera and became the natural spot of the *O.c. toeantei* butterfly.

Currently, the morphological identification of the butterfly are usually based on the wing patterns which solely related to its number and position of points scattered at the wing[5-7]. However, during the development of technology nowadays, it established that the morphological characters identification of lepidoptera species can cause some difficulties in morphometric analysis which could be change as the function of the environment and the prevalence of several biotypes. This condition makes the morphological criteria is no longer the only method chosen for the study of species identification. However, the accuracy of its result has been developed through the molecular based analysis techniques, one of which is the Random Amplified Polymorphic DNA (RAPD) technique and used by Chatterjee and Pradeep[8] to identify the origin of silkworms, *Bombyx mori* L. in India. Analysis of genetic structure of endangered populations in Cranberry Fritillary, Boloria aquilonaris (Lepidoptera, Nymphalidae): with molecular markers of RAPDs vs allozymes by Vandewoestijne and Baguette[9]. Further analysis of genetic diversity with RAPD molecular markers between *Oleria onega agarista* and *Oleria onega* ssp. (Ithomiine, Nymphalidae, Lepidoptera) in Northeast Peru by Gallusser *et al.*[10]. Analysis of genetic relationships between some Lycaenidae butterflies and RAPD molecular markers[11].The use of PCR-RAPD molecular marker techniques is widely used in analyzing butterfly DNA, gene flow between populations, evaluating genetic population structure, determining genetic relationships, phylogenetic and genetic diversity of butterflies both between species and intraspesies[8-12].

Several studies on the genetic variety based on morphological variations of birdwing butterfly are[13] on birdwing butterflies *Trogonoptera* spp., *Troides* spp. and *Ornithoptera* spp.[14] on the macrolepidoptera[15] on the birdwing butterfly at the range of different elevation. Morphometry variation of *Papiliopolytes* butterfly at the upland and lowland of west Sumatra[16]. The butterfly community is generally influenced by habitat conditions, where habitat degradation is more influential than habitat fragmentation[17].

In addition to morphological data that can be analyzed to explain genetic diversity between populations or between species using morphometric data. Data on genetic diversity can also be analyzed using RAPD molecular data[18-21]. The molecular marker PCR-RAPD technique can be used in analyzing DNA polymorphisms, gene flow between populations, between species, evaluation of genetic population structure, determination of genetic relationships, phylogenetics and can also confirm the results of morphological identification based on morphometric characters[22-24].

The RAPD markers are very suitable for use in large samples needed for population genetics and genetic diversity studies[25,26]. The random amplified polymorphic DNA (RAPD) is a PCR-based technique in which random primers inform many regions of genomic DNA. Previously, RAPD was successfully applied for molecular characterization of two species of butterfly familia Pieridae[19], genetic diversity of *O. croesus* butterflies endemic to Bacan Island[27] and genetic variability *O.c. lydius* butterflies endemic to Halmahera Island[28]. This study aims to analyze genetic variability in *O.c. toeantei* butterflies endemic to Morotai Island based on the morphological and molecular characters of PCR-RAPD at various altitudes at Morotai Island as a conservation database for local genetic endemic butterflies. This study describes the feasibility of RAPD in distinguishing *O.c. toeantei* butterflies in 3 altitudes at the similarity of morphological characters.

MATERIALS AND METHODS

Methods: This investigation was conducted in March-April, 2017. For the sampling method, a purposive sampling was used where the sampling technique is free roaming[29].

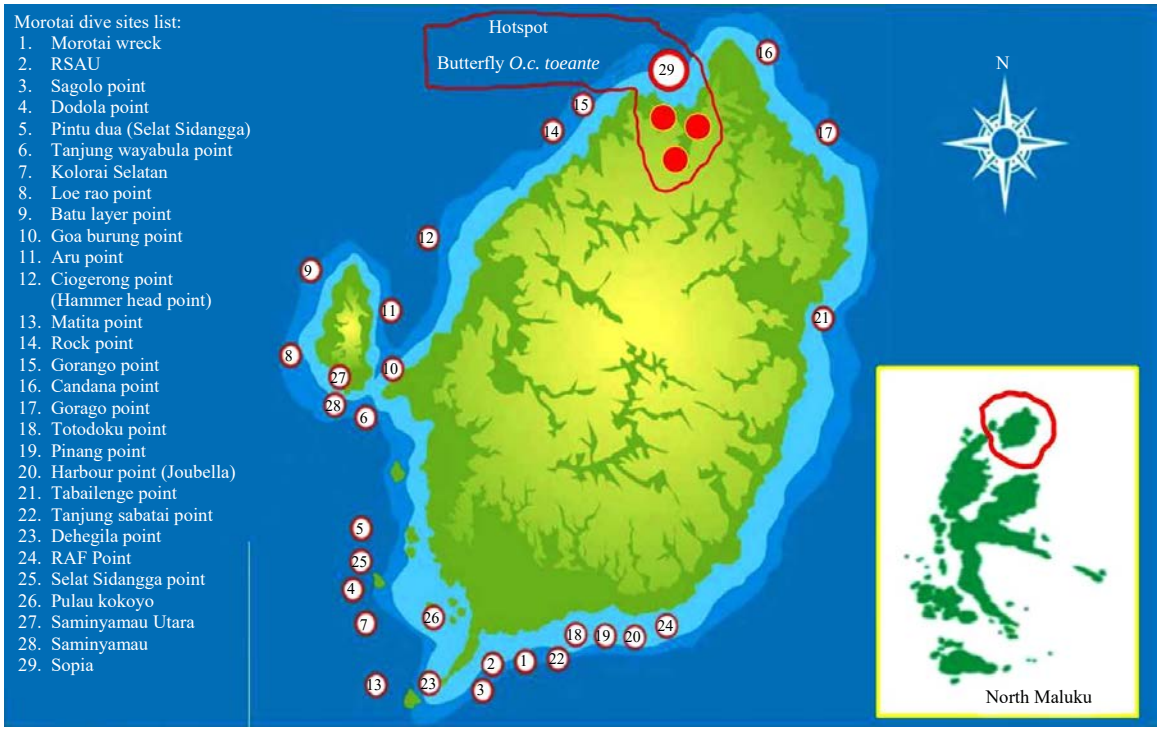

Fig. 1: Research location (hotspot) *O.c. toeantei* on Morotai Island, North Maluku (Morotai Island in Numbers, 2016 and modified by researchers, 2019)

Fig. 2: Species *O.c. toeantei* butterflies endemic to the Island of Morotai

Study area: The collection of *O.c. toeantei* is conducted in 3 different locations of altitude (140 masl, 360 masl, dan 600 masl) in Sopia village Morotai. The map distribution of *O.c. toeantei* is presented in the Fig. 1.

Specimen collection: *Ornithoptera croesus toeantei* butterfly specimens obtained were 24 individuals. The *O.c. toeantei* specimen is preserved by using kaffir (dry matter) to identify morphometric characters. The *O.c. toeantei* specimens were also analyzed molecular-RAPD in the Laboratory of Molecular and Cellular Biology in Brawijaya University as many as 3 male and 3 female individuals (Fig. 2).

Morphometric data measurement: The common standard measurement for the butterfly consist of the measurement of the head length, thoracic length, abdominal length, antenna length, wing length and wing span[7,16,24,30]. In addition to the standard characters in the form of body size, measurements of venation of wings were also carried out.

Measurements on butterfly body size include:

WB : Whole body (measured from the tip of the head to the tip of the abdomen)

LC : Length of caput (measured from the edge of the thorax to the tip of the head)

LTh : Length of thorax (measured from base of the thorax to the border of abdomen)
LA : Length of abdomen (measured from base of the thorax to the tip of the abdomen)
LPbs : Length of proboscis (measured from the base of the mouth or proboscis until the tip part of the proximal proboscis)
LAtn : Length of antenna (measured from the base of the antenna to the tip of the proximal antenna)
LRW : Long range of wings, measured from the front left wing tip to the right front wing tip
LW : Length of wingspan (measured from the left wing tip to the right wing tip)
LFW : Length of front wings (measured from the base of the wing to the tip of the front wing)
LBW : Length of back wings (measured from the base of the back wings to the tip of the back wings)
WFW : Width of the front wings (measured from the mid of upper wing or radius 2 until the mid of lower wing or Cubitus anterior)
WBW : Width of the back wing (measured from the anal vein tip of the back wing until subcostal radius 1 of back wing)
LFL : Length of the front legs (measured from the base of the femur, tibia, tarsus to the nail end of the front leg)
LML : Length of the middle leg (measured from the base of the femur, tibia, tarsus to the tip of middle toe nail)
LBL : Length of the back leg (measured from the base of the femur, tibia, tarsus to the tip of back toe nail)[7,16,24,30]

The measurement of the wing venation, including:

Sc+R1a : Subcostal+Radius 1 of front wing
R2 : Radius 2
R3 : Radius 3
R4 : Radius 4
R5 : Radius 5
M1a : Media 1 of front wing
M2a : Media 2 of front wing
M3a : Media 3 of front wing
CuA1a : Anterior cubitus
CuA2a : Anterior cubitus
A1A2 : Anal veins of front wing
Sc+R1b : Subcostal+Radius 1 of back wing
Rs : Radius sector
M1b : Media 1 of back wing
M2b : Media 2 of wing
M3b : Media 3 of back wing
CuA1b : Anterior cubitus
CuA2b : Anterior cubitus
A1bA2b : Anal veins of back wing

All wing venation characters were measured from the tip of the wing venation to the base of the wing venation[7,16,24,30].

Molecular-RAPD data measurement: The DNA was isolated using the Intron miniprep DNA kit. The DNA amplification at pradenaturation condition at 920°C for 4 min, denaturation at 920°C for 2 min, annealing at 360°C for 1 min 30 sec, extension at 720°C for 2 min and post extension at 720°C for 10 min for 45 cycles by using the Takara PCR with OPA 1 primers to OPA 10 (Table 3). Molecular data is based on the presence or absence of DNA bands with provisions value of 0 for no band and 1 for the presence of DNA bands.

The data obtained from morphometric and molecular measurements were analyzed descriptively quantitatively by using UPGMA cluster analysis (Unweight Pair Group Method with Arithmetic Mean) and the Multivariate Statistical Package (MVSP) program 3.22)[31].

RESULT

Morphometric data: The data of this study is the average data from the morphological characters measurement (quantitative) of Morotai Island endemic butterflies with a total of 42 morphological characters from 24 individuals of *O.c. toeantei* (12 male and 12 female individuals). Then analyzed in similarities between individuals which consisted of analysis of matrix similarities (genetic distance) and dendrogram analysis to explain the diversity of intraspecies *O.c. toeantei*. Data from this analysis for both male and female of *O.c. toeantei* butterflies are shown in Table 1.

In the altitude of 140 masl, *O.c. toeantei* has the highest matrix similarity (coefficient similarity), which is 0.725. This implies that there are most similarities in the morphological character. The lowest matrix similarity (coefficient of similarity) is 0.431 for *O.c. toeantei* ♂ at the altitude of 600 masl and *O.c. toeantei* ♂ at an altitude of 140 masl. This implies that based on its morphological characters, *O.c. toeantei* at different altitude has fewest similarities in morphological characters. Furthermore, based on the results of the matrix similarities as mentioned before a dendrogram can be arranged which shows the group of individuals in the clusters as presented in Fig. 3.

The results of UPGMA analysis (dendrogram) showed that at the similarity value of 0.56, 2 main clusters of *O.c. toeantei*

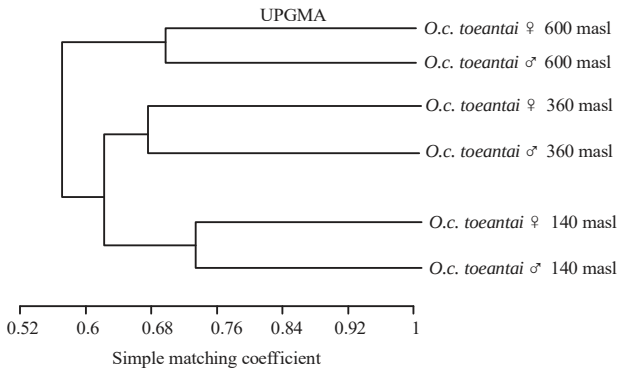

Fig. 3: Dendrogram data of 24 individuals *O. c. toeantei* based on morphological characters analyzed using the UPGMA method through the multivariate statistical package (MVSP) program 32

Table 1: Similarity data of morphological characters *O.c. toeantei* Morotai Island endemic butterfly based on morphological characters analyzed using UPGMA cluster

	Orinithoptera croesus toeantai					
Morphological characters	♂ (140 masl)	♀(140 masl)	♂ (360 masl)	♀(360 masl)	♂ (600 masl)	♀(600 masl)
O.c. toeantai ♂ (140 masl)	1					
O.c. toeantai ♀ (140 masl)	0.725	1				
O.c. toeantai ♂ (360 masl)	0.588	0.644	1			
O.c. toeantai ♀ (360 masl)	0.600	0.619	0.669	1		
O.c. toeantai ♂ (600 masl)	0.431	0.606	0.563	0.544	1	
O.c. toeantai ♀ (600 masl)	0.625	0.581	0.581	0.613	0.694	1

♂: Male, ♀: Female

Table 2: RAPD primary data sequence and polymorphic (%) *O.c. toeantei* based on the presence of the DNA-RAPD band pattern analyzed by the UPGMA method

Primers	Seq 5-3	Seq 5-3 bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)
OPA-1	CAG GCC CTT C	7	7	0	100.00
OPA-2	TGC CGA GCT G	9	7	2	77.77
OPA-3	AGT CAG CCA C	7	6	1	85.71
OPA-4	AAT CGG GCT G	8	6	2	75.00
OPA-5	AGG GGT CTT G	4	2	2	50.00
OPA-6	GGT CCC TGA C	6	5	1	83.33
OPA-7	GAA ACG GGT G	5	4	1	80.00
OPA-8	GTG ACG TAG G	5	3	2	60.00
OPA-9	GGG TAA CGC C	4	3	0	75.00
OPA-10	GTG ATC GCA G	5	4	1	80.00
Total		60	47	12	76.681

at an altitudes of 140 masl, 360 masl and 600 masl were formed. The main cluster I with a similarity value of 0.70 consisted of *O.c. toeantei* ♀ 600 masl and *O.c. toeantei* ♂ 600 masl. The main cluster II with a similarity value of 0.62 formed into 2 subclusters, namely subcluster I with a similarity value of 0.67 consisting of *O.c. toeantei* ♀ 360 masl and *O.c. toeantei* ♂ 360 masl and subcluster II with a similarity value of 0.72 consisting of *O.c. toeantei* ♀ 140 masl and *O.c. toeantei* ♂ 140 masl. Thus, it is known that the most morphological characters are found on *O.c. toeantei* at the altitude of 140 masl and the lowest similarity at the altitude of 360 masl.

PCR-RAPD molecular data: The data of Molecular-RAPD character is achieved from the calculation of DNA bands appearance of *O.c. toeantei* with the total sample of 6 individuals (3 male and 3 female individuals) at different altitudes (140 masl, 360 masl and 600 masl) (Table 2).

The total bands resulting from this study are 60 bands with criteria of 42 of polymorphic and 18 of monomorphic. Those results were identified from the DNA's band pattern appeared from the DNA photo. The average percentage of polymorphic is 76, 246% on OPA 1-10 primer (Table 3). Next, a further analysis was conducted on the matrix similarity based on the appearance of DNA band (DNA profile) with

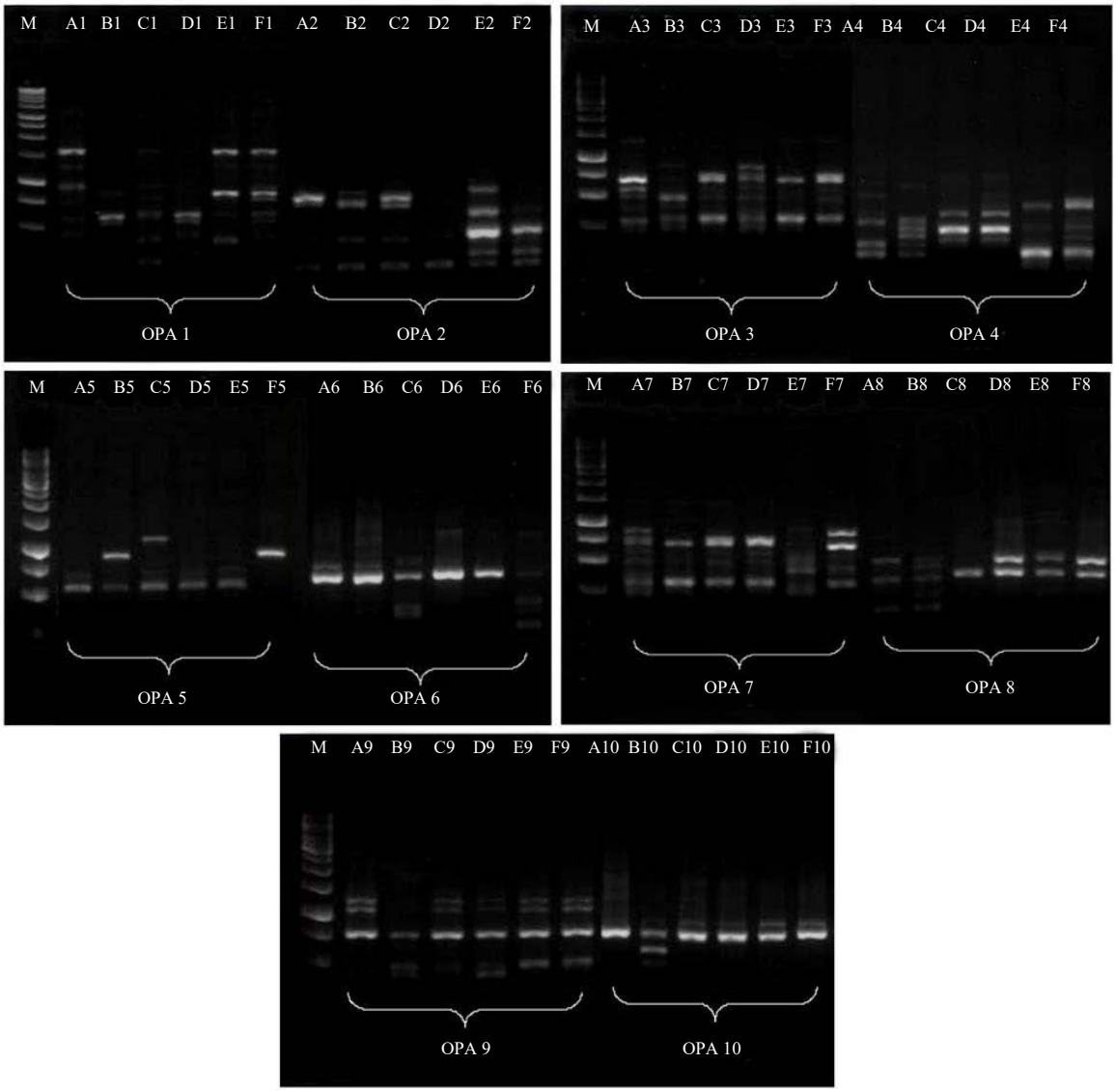

Fig. 4: Visualization of RAPD band pattern of 6 individuals *O.c. toeantei* with OPA primer 1-10

Table 3: Similarity of *O.c. toeantei* matrix based on the presence of the DNA-RAPD tape pattern analyzed by the UPGMA method

	Orinithoptera croesus toeantai					
Morphological characters	♂ (140 masl)	♀(140 masl)	♂ (360 masl)	♀(360 masl)	♂ (600 masl)	♀(600 masl)
O.c. toeantai ♂ (140 masl)	1					
O.c. toeantai ♀ (140 masl)	0.767	1				
O.c. toeantai ♂ (360 masl)	0.600	0.567	1			
O.c. toeantai ♀ (360 masl)	0.600	0.533	0.733	1		
O.c. toeantai ♂ (600 masl)	0.667	0.533	0.633	0.667	1	
O.c. toeantai ♀ (600 masl)	0.583	0.517	0.583	0.683	0.750	1

♂: Male, ♀: Female)

1 as the score for DNA band appearance and 0 if the DNA band does not appears in every OPA 1-10 primer (Fig. 4).

The results of the calculation of the appearance of DNA bands from the visualization of DNA band profiles (Fig. 4) can be analyzed the similarity of the matrix *O.c. toeantei* with the UPGMA method as in Table 3.

In the highest value of matrix similarity (coefficient similarity) is 0.767 at altitude of 140 masl. This implies that

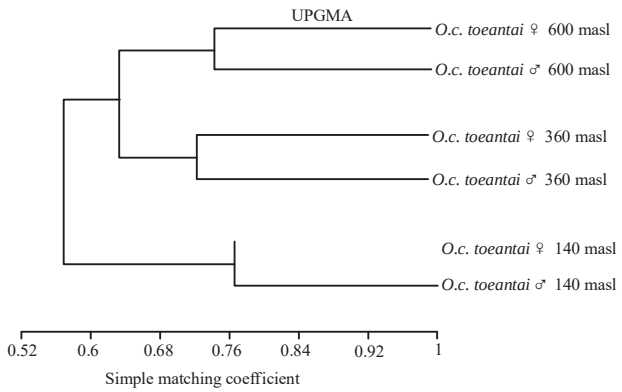

Fig. 5: Dendrogram data of 8 individuals *O.c. toeantei* based on the presence of DNA-RAPD band patterns analyzed by UPGMA method

there is a high similarity between *O.c. toeantei* ♂ and *O.c. toeantei* ♀ based on RAPD analysis. The lowest value of matrix similarity is 0.517 at altitude of 140 masl and 600 masl. This, implies that the DNA appearance on the RAPD analysis has the lowest similarity, in which for *O.c. toeantei* ♀ at the altitude of 140 masl and for *O.c. toeantei* ♀ at the altitude of 600 masl. The dendrogram resulted from average matrix similarity 10 primer OPA can be seen on the Fig. 5.

The result from UPGMA (dendrogram) analysis showed that on the similarity value of 0.57, 2 main cluster of *O.c. toeantei* at the altitudes of 140 masl, 360 masl and 600 masl were formed. The I main cluster with the similarity value of 0.63 formed into 2 sub clusters consisting of 1 main sub cluster with similarity value of 0.72 that are *O.c. toeantei* ♀ 360 masl and *O.c. toeantei* ♂ 360 masl. 2 main sub clusters with similarity value of 0.77 that are *O.c. toeantei* ♀ 140 masl and *O.c. toeantei* ♂ 140 masl. Thus, it is known that the more character similarity in morphology is found on *O.c. toeantei* at the altitude of 140 masl and the lowest similarity at the altitude of 360 masl.

DISCUSSION

The result of this study showed that there are similarity on the clustering pattern of morphological and molecular-RAPD character dendrograms of *O.c. toeantei* at different level of altitude on Morotai Island. The most high similarity was found at the location of 140 masl of altitude (Table 2). This, became the evidence that morphological variability of the *O.c. toeantei* butterfly can be influenced by the different of altitude. The sex on the other hand, did not shows any influence to the variation of morphometry and molecular-RAPD, however its only visible on color's variation at the body and wings. The result of this investigation also showed that the Morotai Island has the high categorize of *O.c. toeantei* intraspecies variations. It can be seen from its lowest value of similarity in both morphometry and molecular-RAPD (Table 1 and 3). Furthermore, from the cluster UPMGA analysis (Fig. 2 and 3) its been known that the similarity value between the main cluster were 0.56 on the morphometry and 0.56 on the molecular-RAPD. This shows that the similarity between cluster is low at morphological as well as molecular-RAPD.

The similarity of morphological and molecular-RAPD character that appears at certain level of altitude indicates the different habitat of *O.c. toeantei*. One of the main factor of this clustering pattern is the food abundance (mussaenda and ashoka). This is in line with the study of Koneri and Saroyo[17] who stated that based on the type of the butterfly, the variation and abundance of the species is tend to be high at low part than the high part of the area which related to availability of the food. Moreover[24] stated that the amount of food at the high level of area was very limited even none for *Ornithoptera* spp., while at the lower part have the high amount of it Van Vu and Vu[32], Van Lien and Yuan[33] and Dendang[34] stated that the more high the variety of vegetation the more high the variety of butterfly.

The result from UPGMA on the morphological and molecular-RAPD dendrograms (Fig. 4 and 5) explains that each dendrogram formed into 2 main cluster which organized at each level of altitude. The main similarity value of morphological character dendrogram is 0.56 which also the same at molecular-RAPD character dendrogram. This data showed an in line condition at the morphological and molecular-RAPD characterization. The similarity value of *O.c. toeantei* an endemic butterfly of Morotai Island falls into low category which below 80%[35]. The low of similarity values implies the high of diversity. The similar study by Mas'ud[24] on the genetic diversity of *Ornithoptera croesus* reported that

the genetic diversity is correlated positively to the high of hotspot level. Variational genetic studies of two Cirrochroa using the RAPD-PCR technique conducted by Zothansangi *et al.*[21], showed that there are genetic variations with the similarity of the two cryptic species. The RAPD technique that can determine molecular characterization which has morphological similarities to four morphologically similar butterflies similar to Pieridae[20].

Dendrogram construction provides the evidence that intraspecific variation (genetic variation) at the molecular-RAPD level between *O.c. toeantei* ecotype. The dendrogram results (Fig. 4) based on molecular-RAPD data shows a number similarities with the morphological differentiation of 2 main clusters and 2 subcluster of *O.c. toeantei* from different level of altitude. The clustering pattern of the 2 main clusters is supported by morphological similarities of 2 subclusters. The main cluster and subcluster patterns strongly support morphological characters. The RAPD technique has revolutionized the field of molecular biology for the study of butterfly DNA, gene flow between populations, evaluation of genetic population structures, determination of genetic relationships and phylogenetics. Likewise in the study of genetic diversity of *O.c. toeantei* butterfly an endemic from Morotai Island.

This research is the first attempt to conserve endemic butterflies on the Morotai Island of North Maluku carried out at the level of intraspecies diversity of *O.c. toeantei* based on morphological and molecular characters. This research was also carried out as an effort to preserve the endemic butterfly of North Maluku, Indonesia. Butterfly is a genetic resource that needs to be preserved as a pollinator and biological wealth which has ecological and economic value.

CONCLUSION

There is a similarity in the clustering pattern between UPGMA analysis on morphometric and molecular-RPAD characters of *O.c. toeantei* the endemic butterfly of Morotai Island. The main similarity values of the UPGMA analysis were 0.56 in the morphometric dendrogram and 0.56 in the molecular-RAPD dendrogram. This information shows that the *O.c. toeantei* has a high similarity on the habitat with the same altitude, whereas in habitats with different altitude the similarity tends to low. In general, *O.c. toeantei* the endemic butterflies of Morotai has a high genetic diversity at the intraspecies level.

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