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Research Article

Clustering Analysis and Genome Inference of Pisang Raja Local Cultivars (*Musa* spp.) from Java Island by *Random Amplified Polymorphic DNA* (RAPD) Marker

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ABSTRACT

Pisang Raja is an important local banana cultivar in the economy and cultural life in Indonesia, especially at Java. There are many Pisang Raja cultivars found on Java Island with various local names in each region, resulted in problems on taxonomic identification and grouping. Conventional research for grouping banana cultivars is still using morphological characters but considered inaccurate because of its subjectivity. This study aims to analyze the genetic diversity, grouping, and genome estimation of 13 local cultivars of Pisang Raja based on molecular approach using RAPD markers (OPA primers 1-20). Clustering and Principal Coordinates Analysis were performed to the amplified products using Paleontological Statistics (PAST) application version 3.15. Results showed that there were 12 primers which successfully amplified and produced DNA polymorphic bands in Pisang Raja, specifically OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20. Pisang Raja cultivars considered have high genetic diversity, indicated by high polymorphic bands (95.17%) and low similarity coefficient values (0.2-0.6). Clustering and PCo analysis resulted in 3 clusters following its genomic group consist of AAA, AAB and ABB genomes, with Pisang Raja Bali as an outgroup (ABB). However, the separation of each cluster for genome inference was unclear. Cluster 1 consists of Pisang Raja Madu (AAB) and Raja Sereh (AAB). Cluster 2 consists of AAA and AAB genomes; includes Pisang Raja Jambe (AAA), Raja Kriyak (AAA), Raja Kutuk (AAB), Raja Brentel (AAB), Raja Seribu (AAB), and Raja Lini (AAB). Cluster 3 consists of AAA and AAB genomes, includes Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Bandung (AAB) and Raja Gareng (AAB). While Pisang Monyet (AAw) and Klutuk Wulung (BBw) as wild relatives were nested in Cluster 2. There were some different results of genome estimation based on RAPD markers compared to morphological characterization, and other molecular techniques. The use of RAPD markers is quite efficient and effective for studying genetic diversity and identifying genomes in bananas.

INTRODUCTION

Bananas (*Musa* spp.) are herbaceous plants in the Musaceae family which widely distributed in tropical and subtropical regions (Simmonds, 1959). Indo-Malesia is the origin center of banana diversity in the world, which then spread to all tropical and subtropical regions in Asia, America, Africa and Australia. (Simmonds and Shepherd, 1955; De

Langhe *et al*., 2009). In Indonesia, the diversity center of bananas is spread across Sulawesi, Sumatra, Madura and including Java (Ochse, 1931). Nowadays, bananas are fruit plant with high value as a horticultural commodity in the world (Ministry of Agriculture, 2016). Indonesia has contributed 5.67% and occupies the sixth position as the world's banana production center. Some regions which contributed to banana production in Indonesia, include East Java (21.87%) West Java (19.22%), Lampung (18.20%), and Central Java (7.68%) (Ministry of Agriculture, 2016). The number of local bananas available in Indonesia is more than 200 cultivars. Some of the popular banana cultivars in Indonesia, viz. Pisang Susu, Kepok, Tanduk, Nangka, Ambon, Raja, etc. (Nasution and Yamada, 2001).

Pisang Raja is one of the popular banana which has an important role in the economy and cultural life in Indonesia, especially at Java Island (Hapsari *et al.,* 2017). In Indonesia, Pisang Raja is widely produced in Java Island, such as in West Java (Sukabumi and Cianjur), Central Java (Demak) and East Java (Lumajang) (Ministry of Agriculture, 2016). There were not less than 40 Pisang Raja local cultivars recorded from several studies of inventory and diversity of bananas in Java Island (Jumari and Pudjoarinto, 2000; Indraswari, 2014; Firdausi *et al.,* 2015; Hapsari *et al.,* 2017; Hapsari *et al.,* 2018). The use of different local names in each region is a problem in the taxonomic nomenclature of Pisang Raja. For example, Pisang Raja Bulu was also known as Raja Madu in Jember, Raja Kul was also known as Raja Talun in Pasuruan and Probolinggo, Raja Pakak was also known as Raja Sepet in Pasuruan, and Raja Sajen was also known as Raja Talun in Malang (Hapsari *et al.,* 2017).

Pisang Raja is a species of hybridized plant which generally has triploid genome characters of AAB, AAA and ABB (Espino *et al*., 1992; Jumari and Pudjoarinto, 2000; Ekasari *et al*., 2012; Hapsari *et al*., 2015a). Those genome characters owned by Pisang Raja was originated from ancestors *Musa acuminata* Colla (A genome contributor) and *Musa balbisiana* Colla (B genome contributor). Further, to overcome the problem of many local names in banana cultivars, Simmonds and Shepherd (1955) proposed the use of genome nomenclature was then approved by a consensus in 1999, which consist of generic name, followed by letter combinations indicating the ploidy and genome sets contributed by their ancestral, followed by the name of cultivars group or the cultivars (Simmonds, 1959; Valmayor *et al*., 2000; INIBAP, 2006).

Genomic composition and various ploidy levels of banana cultivars were first derived from the development of parthenocarpy and sterility. Those events were followed by chromosome restitution and out-crossing of the ancestral both intra and inter -species, then high bananas diversity emerged with various ploidy levels and genomic combination such as AA, AAA, AB, AAB, BB, and ABBB (Simmonds, 1959; Espino *et al*., 1992; Singh *et al*., 2001; Valmayor *et al*., 2000). In determining the genome of banana cultivars can be done by scoring assessment based on morphological characters (Simmonds and Shepherd, 1955; Simmonds, 1959). However, morphological approaches are often inaccurate because it was subjective and can be influenced by environmental factors (Jumari & Pudjoarinto, 2000; Guzow-Krzeminsk *et al*., 2001). Thus, it is necessary to conduct grouping and taxonomy nomenclature analysis based on genotypes with molecular markers.

The approaches through molecular markers were known to have a higher level of accuracy compared to the morphological approach in the identification and grouping of banana cultivars (Williams *et al.,* 1990; Maftuchah, 2001; Rao and Hodgkin, 2002; de Jesus *et al.,* 2013; Hapsari *et al.,* 2015a). One of the molecular markers which often used in research on genetic diversity in plants was *Random Amplified Polymorphic* DNA (RAPD). It is a DNA amplification technique based on *Polymerase Chain Reaction* (PCR) using random single oligonucleotides (primers) to form DNA fragments (Bustaman and Moeljopawiro, 1998; Dayarani and Dhanarajan, 2014). Further, RAPD markers are often used to determine the taxonomy and grouping on banana cultivars in many countries (Uma *et al.,* 2006; Sukartini, 2008; Brown *et al.,* 2009; Olivia *et al.,* 2010; Makunthakumar *et al.,* 2013; Kiran *et al.,* 2015; Nair, 2016). This method has several advantages in the simplicity of technique, fast process, only requires a small amount of DNA samples (0.5-50 ng), and no need initial genome information (Demeke and Adams, 1994; Yu and Pauls, 1994).

The present study aims to analyze the genetic diversity, grouping and genome inference of Pisang Raja local cultivars from several regions of Java Island based on RAPD molecular markers. The results of this study are expected to support the process of characterization and genome identification of Pisang Raja more accurately, which then can be used as information to confirm and evaluate the taxonomy nomenclature of Pisang Raja. It is also expected to be used as basic information for further researches related to conservation, breeding and development of Pisang Raja particularly in Java Island as valuable genetic resources.

Table 1. Samples of banana cultivars used in this study. R1-R13 = in-group, AA3 and BB1 = outgroup.

No.	Local name	Genome*	Sample code	Collection site in Java Island
1	Raja Bali	ABB	R1	Bantul, Central Java
2	Raja Bandung	ABB	R2	Bantul, Central Java
3	Raja Delima	AAA	R3	Malang, East Java
4	Raja Kisto	AAA	R4	Banyuwangi, East Java
5	Raja Gareng	AAB	R5	Temanggung, East Java
6	Raja Jambe	AAA	R6	Malang, East Java
7	Raja Kriyak	AAA	R7	Temanggung, Central Java
8	Raja Madu	AAA	R8	Banyuwangi, East Java
9	Raja Sereh	AAB	R9	Purworejo, Central Java
10	Raja Seribu	AAB	R10	Jakarta
11	Raja Kutuk / klutuk	AAB	R11	Purworejo, Central Java
12	Raja Lini	AAB	R12	Sukoharjo, Central Java
13	Raja Brentel	ABB	R13	Gunung Kidul, Central Java
14	Monyet (*M. acuminata*)	AAw	AA3	Tuban, East Java**
15	Kluthuk wulung (*M. balbisiana*)	BBw	BB1	Banyumas, Central Java

* genome identity based on morphology
** collection of Purwodadi Botanic Garden

MATERIALS AND METHODS

Materials

Thirteen local cultivars of Pisang Raja and two wild bananas (Table 1) were used in this study. It was collected from Banana Germplasm Garden of Yogyakarta, at Special Region of Yogyakarta. Pisang Raja cultivars were originated from several districts of Java Island, consisting of 8 cultivars from Central Java, 4 cultivars from East Java, and 1 cultivar from Jakarta (Table 1). The genomic group of each Pisang Raja cultivar was previously identified morphologically based on minimal descriptor for bananas (Simmonds, 1959), according to information from the curators, and also from some references (Jumari & Pudjoarinto, 2000; Sukartini, 2007; Wahyuningtyas, 2009; Hapsari, 2014; Hapsari *et al.*, 2015b, Nedha *et al.*, 2017). It comprised of AAA, AAB and ABB genomes (Table 1). In addition, wild bananas of *M. acuminata* (AAw) and *M. balbisiana* (BBw) species were also analyzed for comparison as outgroups. Further, the plant material used was young leaves (furled) which dried with *silica gel* prior to analysis, one sample per cultivar.

DNA Isolation

DNA isolation of thirteen samples was carried out using DNA genome purification kit from the Promega Wizard®, following its manufacturer's procedure for plants. Then, the total genome isolated were confirmed both by quantitative and qualitative tests to determine DNA concentration and purity. Quantitative test with determination of absorbance value at length 260 nm and 280 nm was performed using AE-Nano 200 Nucleic Acid Analyze version 2.0. Good DNA purity level indicated by the value of the *Optical Density* (OD) 260/280 nm ratio approximately 1.8-2.0 (Sambrook *et al.,* 1989). Whilst, the qualitative tests were performed using electrophoresis on 1% agarose gel with the addition of 2 µg/ml *Ethidium bromide* (Etbr) for 30 minutes at a voltage of 80 volts, then photographed on GelDOC UV – Transiluminator (BioRAD). The estimated length of total genomic DNA was measured using a 1-Kb DNA *ladder* markers (Thermo Scientific, California, USA).

RAPD - PCR Analysis

DNA amplification was conducted with PCR Thermocycler using 20 selected RAPD primers from Operon Technology Ltd (Table 2). The PCR reaction mixture was performed with a total volume of 10 µl consisting of 1 µl DNA template (5-25 ng/µl), 1 µl primer OPA 1-20 (10 pmol), 3 µl ddH_2O, and 5 µl PCR master mix (Thermo Scientific, California, USA). The PCR cycle consists of DNA pre-denaturation at 94 °C for 4 minutes, then 45 cycles consist of denaturation at 94 °C for 30 seconds, annealing at different temperatures for each primer according to Table 2 for 30 seconds, extension at 72 °C for 30 seconds and followed by post elongation at 72 °C for 5 minutes. PCR products were then visualized in 1.5% agarose gel electrophoresis in TBE buffer 1X, 2 µl EtBr for 50 minutes at 50 volts and documented with UV transillumilator. DNA ladder 100 bp (Thermo Scientific, California, USA) was used to determine the size of DNA amplification fragments.

Table 2. List of RAPD primers used in this study (OPA 1- OPA 20). MT = melting temperature, A = Adenine, T = Thymine, G = Guanine, and C = Cytosine.

Primer code	Primer nucleotide sequence (5'-3')	MT (°C)	Annealing (°C)	GC composition (%)
OPA-01	5' - CAG GCC CTT C – 3'	36.4	41	70
OPA-02	5' - TGC CGA GCT G – 3'	40.7	45	70
OPA-03	5' - AGT CAG CCA C – 3'	34.3	39	60
OPA-04	5' - AAT CGG GCT G – 3'	35.1	40	60
OPA-05	5' - AGG GGT CTT G– 3'	32.6	37	60
OPA-06	5' - GGT CCC TGA C– 3'	35.2	40	60
OPA-07	5' - GAA ACG GGT G – 3'	33.2	38	60
OPA-08	5' - GTG ACG TAG G – 3'	31.1	36	60
OPA-09	5' - GGG TAA CGC C – 3'	37.4	42	70
OPA-10	5' - GTG ATC GCA G – 3'	33.1	38	60
OPA-11	5' - CAA TCG CCG T – 3'	36.7	41	60
OPA-12	5' - TCG GCG ATA G – 3'	34.0	39	60
OPA-13	5' - CAG CAC CCA C – 3'	37.7	42	70
OPA-14	5' - TCT GTG CTG G – 3'	34.3	39	60
OPA-15	5' - TTC CGA ACC C – 3'	34.2	39	60
OPA-16	5' - AGC CAG CGA A – 3'	38.3	43	60
OPA-17	5' - GAC CGC TTG T – 3'	35.7	40	60
OPA-18	5' - AGG TGA CCG T – 3'	36.2	41	60
OPA-19	5' - CAA ACG TCG G – 3'	34.2	39	60
OPA-20	5' - GTT GCG ATC C – 3'	33.5	38	60

Data Analysis

RAPD molecular data were analyzed based on amplified DNA band products that appeared on the gel by scoring prior to analysis to determine polymorphism. RAPD products which reproducible, well resolved and non-ambiguous were scored manually as '1' for presence and '0' for absence of a fragment. The binary data matrix was tabulated for further analysis. Polymorphism analysis and discriminatory power of each primer were evaluated by means of four parameters include polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolution power (RP) (Laurentin and Karlovsky, 2007).

Cluster analysis to determine the grouping pattern and genome inference of Pisang Raja was calculated by Jaccard's coefficient similarity, using PAST application version 3.15. Multivariate ordination analysis of the principal coordinate analysis (PCoA) also conducted by PAST program with menu options: multivariate ordination-principal coordinates analysis, matrix eigenvalues, and eigenvectors (Hammer *et al.*, 2001). Further, the results of genome inference from RAPD analysis were compared to morphological characters and other molecular methods obtained from previous researches and references.

RESULTS AND DISCUSSION

RAPD profiles Pisang Raja

Results of DNA amplification showed that 12 out of 20 RAPD primers had the capability to produced polymorphic bands of Pisang Raja, specifically OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 11, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20. However, some samples of Pisang Raja have not produced any bands or absent on certain primers (Figure 1b-d-f). It may due to the absence of homolog primer sequences information in the genome. The number of DNA amplification bands depended on how primer attached to its homolog at DNA template which noted by the presence or absence of an amplification product from a single locus (Tingey et al. 1994). In addition, it may also have caused by technical errors, amplification process and thermal cycle which were less suitable of certain primers for certain sample. Further analysis for those certain primers and samples of Pisang Raja are required to conduct.

Each pattern of DNA band amplification products is an informative character for describing the construction of genetic diversity for genetic relationships among samples. The amplification products showed high polymorphic bands in 13 Pisang Raja cultivars. It comprised 4.83% monomorphic and 95.17% polymorphic bands. Monomorphic bands that emerged showed no variation in all samples, for example at the primers OPA 1 (800 bp), OPA 4 (400 bp) and OPA 11 (1500

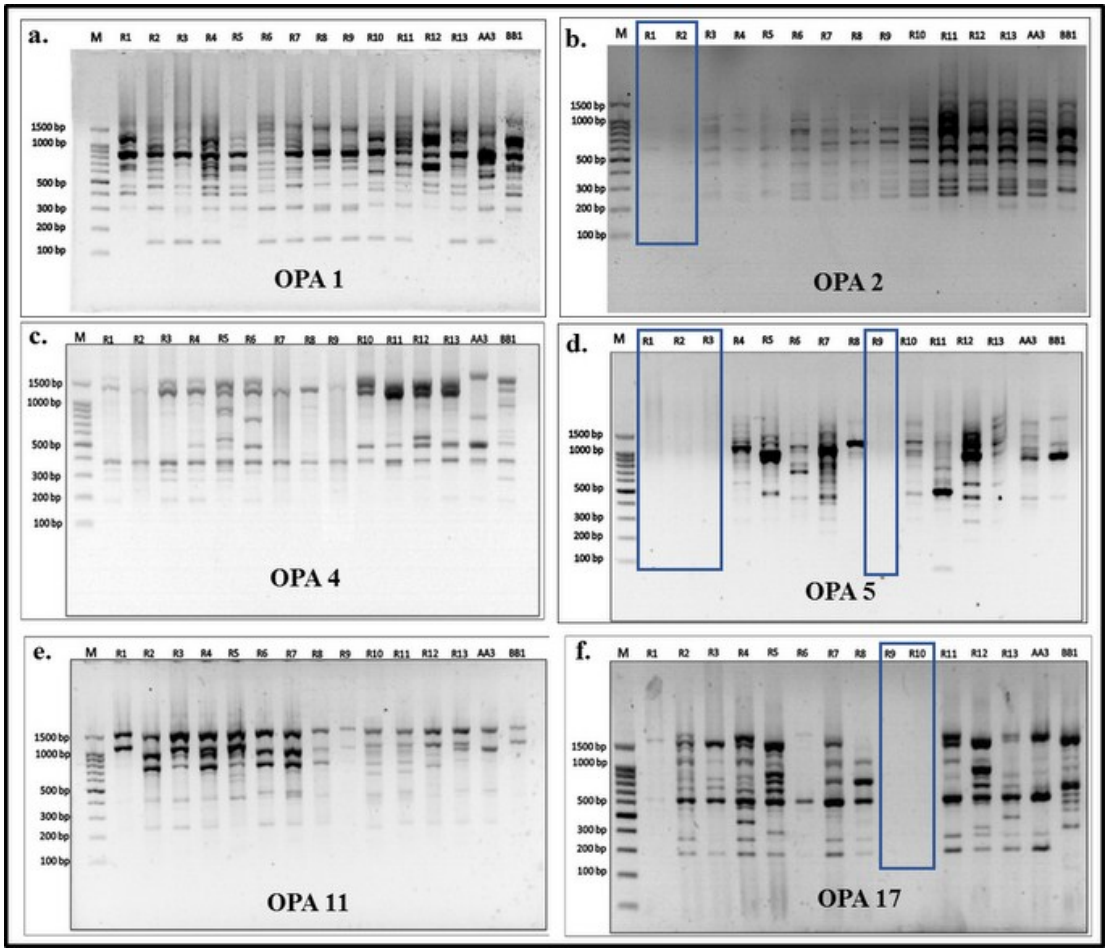

Figure 1. Electrophoregram PCR RAPD which produced DNA polymorphic bands on Pisang Raja. Blue rectangle: showed an absence of DNA band in certain primers and samples.

bp) (Figure 1a-c-e). Those monomorphic bands were presumably considered as part of some genes which encode for the same characters in all Pisang Raja samples.

Further, RAPD primers which produced the highest number of polymorphic bands were OPA 2 (11 bands) and OPA 17 (11 bands), while the lowest polymorphic bands were OPA 1 (8 bands) and OPA 19 (8 bands) (Table 3). RAPD polymorphism is the result of either a nucleotide base change that alters the primer binding site or an insertion or deletion within the amplified region (Williams *et al.* 1990). The differences in polymorphism may be due to the differences in amount of genetic variation that exist among the different accessions (Poerba and Achmad, 2010). Further, the appearance of high polymorphic bands on PCR amplification products indicated that the genetic diversity of the species examined was high (Roldan-Ruiz *et al.*, 2000). Thus, this study provides evidence which indicates that 13 Pisang Raja cultivars examined have high genetic diversity.

Results of polymorphism analysis

The total number of bands (TNB) DNA fragments produced by 12 RAPD primers on 13 Pisang Raja cultivars were 121 bands with sizes ranging from 100 bp to 1500 bp. The highest TNB appeared was produced by OPA 2, OPA 4, and OPA 17; each with 11 bands. Of the 121 total bands produced, 115 of them are considered polymorphic bands. The highest number of polymorphic bands (NPB) produced by OPA 2 and OPA 17 were 11 bands (Table 3). The polymorphic band percentage (PB%) ranged from 80% -100%. Due to their high reproducibility, this result indicated that those 12 primers are suitable to be used as markers for detecting genetic diversity in Pisang Raja. Hence, those 12 RAPD primers (OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 11, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20) are proposed as suitable primers for similar research to study the genetic diversity and infer the genome of bananas.

Polymorphism information content (PIC) is information to detect primers which capable of producing polymorphic bands in a population (Roldan-Ruiz *et al.*, 2000). The highest PIC value was indicated by OPA 2 and OPA 17 primers of 0.34, whereas the lowest PIC was indicated by OPA 16 primer of 0.15 (Table 3). PIC maximum value for RAPD markers was 0.5. PIC values were used to

Table 3. Polymorphism analysis results of OPA 1-20 RAPD primers amplification in Pisang. TNB = total number of bands; NPB = number of polymorphic bands; PB = polymorphic band percentage; PIC = polymorphism information content; EMR = effective multiplex ratio; MI = marker index; RP = resolution power.

No	Primer name	TNB	NPB	PB (%)	PIC	EMR	MI	Rp
1.	OPA 1	10	8	80	0.21	80	17.70	15.60
2.	OPA 2	11	11	100	0.34	121	41.02	11.33
3.	OPA 3	10	9	90	0.25	90	22.86	13.07
4.	OPA 4	11	9	82	0.22	99	21.75	9.33
5.	OPA 5	10	10	100	0.27	100	26.54	6.53
6.	OPA 8	9	9	100	0.25	81	20.57	5.07
7.	OPA 11	10	9	90	0.25	90	22.40	10.27
8.	OPA 16	7	7	100	0.15	49	7.40	2.67
9.	OPA 17	11	11	100	0.34	121	40.87	12.80
10.	OPA 18	10	10	100	0.31	100	30.86	8.67
11.	OPA 19	8	8	100	0.20	64	12.52	4.80
12.	OPA 20	9	9	100	0.28	81	22.32	8.00
	Total	121	115	1142	3.19	1155	328.11	117.74
	Mean	10.10	9.58	95.17	0.27	96.25	27.34	9.81

consider which the best primer in RAPD markers and reflects allele diversity and frequency among samples. The higher PIC value means the better of the primer to be used to analyze genetic variation (Roldan-Ruiz *et al*., 2000).

Furthermore, the highest effective multiplex ratio (EMR) value was observed in OPA 2 and OPA 17 (121), and the lowest was observed in OPA 16 (49) (Table 3). EMR analysis was performed to determine the effective ratio of the number of bands produced with the number of polymorphic bands (Roldan-Ruiz *et al*, 2000). While, the OPA 2 primer has the highest marker index (MI) of 41.02, and OPA 16 has the lowest MI value of 7.40. The marker index (MI) value was used to estimate the usefulness of markers in practical terms results in a total band value that appears proportional to the number of polymorphism bands (Varshney *et. al*., 2007). Resolution power (Rp) analysis was used to determine the effectiveness of the primer of produced bands. Each primer has a value of RP which ranged from 2.67-15.60 with an average of 9.81 per primer. The highest RP value was generated by OPA 1 primer with a value of 15.60 while the lowest value was found in OPA 16 primer with a value of 2.67 (Table 3). Based on the overall polymorphism parameters analyzed, the most effective primer in producing polymorphic bands in Pisang Raja was OPA 2 and OPA 17.

Clustering and genome inference of Pisang Raja in Java Island by RAPD marker

Genetic similarity analysis showed that among 13 Pisang Raja cultivars in Java Island has high genetic diversity with similarity coefficient ranged of 0.22-0.61. Low similarity coefficient indicates that the genetic relationship among cultivars observed has high genetic diversity, vice versa. The lowest similarity coefficient was obtained among Pisang Raja Bali (ABB) and Raja Seribu (AAB). They both have different genomes and collected from different provinces *i.e.* Central Java and DKI Jakarta, respectively. Whilst, the highest similarity coefficient was observed between Pisang Raja Kutuk (AAB) and Raja Brentel (AAB), they shared high genetic identity at 0.61 similarities. They both have the same genomes and similar morphological characteristics also collected from the same province, i.e. Central Java.

The conventional classification of banana genotypes into distinct genome combinations by Simmonds and Shepherd (1955) is basically according to their morphological similarity to *M. acuminata* and *M. balbisiana*. The 13 Pisang Raja cultivars examined in this study using RAPD bands profiles resulted a dendogram which may give a picture of genetic relationship and taxonomic position, also genomic group inference of each cultivar which clustered accordingly to their hypothetical genetic homologies. The dendogram was separated into three main clusters at a genetic similarity coefficient of 0.39 (Figure 2). However, the deeper separation of each cluster for genome inference was unclear. It may due to the intensity and number of the amplified DNA bands were less consistent and reproducible on some samples. The first amplification results do not always produce a band with the same intensity at the next amplification. Amplification of each RAPD primer is strongly influenced by the primer attachment site in the DNA template. RAPD primer has amplified whole genomic DNA template, it is possible for primers attachment to be initiated in several places (randomly), but only a few sets can be detected as

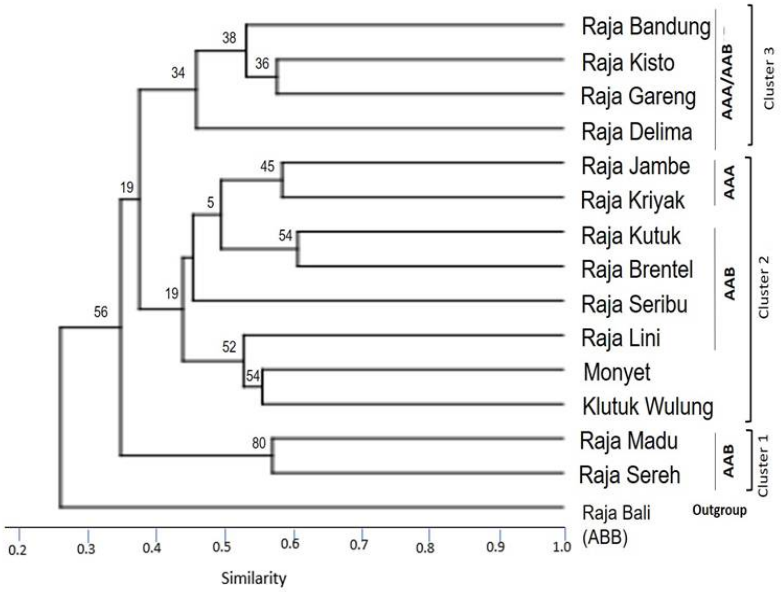

Figure 2. Dendrogram grouping pattern of Pisang Raja and genome inference based on RAPD marker

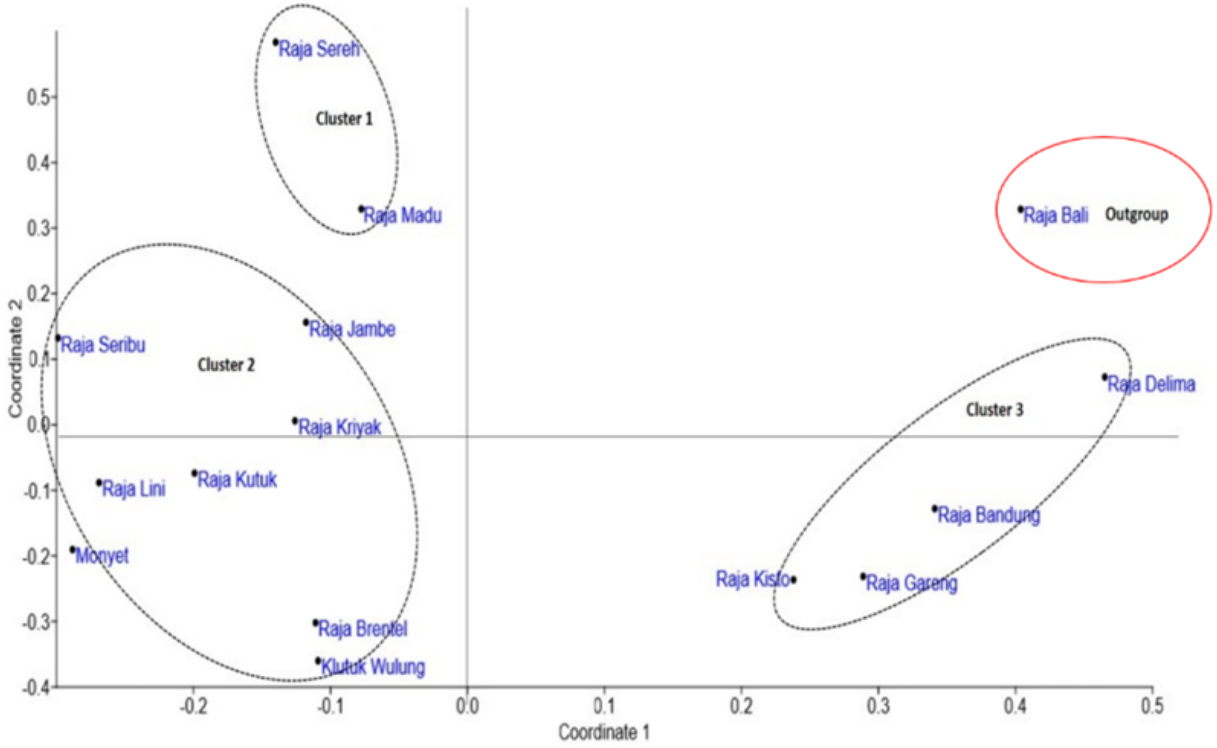

Figure 3. Principal Coordinate (Pco) scatter plot of Pisang Raja based on RAPD marker

after amplification band (Williams *et al*., 1990). As a result, the polymorphic DNA bands produced by each primer differed in the second amplification, both in the size of the number of base pairs and the number of DNA bands, thus affecting the accuracy in grouping banana cultivars with the same genome (Poerba and Ahmad, 2013; Sukartini, 2008).

Cluster 1 consists of Pisang Raja Madu and Raja Sereh which have AAB genomes, with a similarity coefficient of 0.58. Cluster 2 consists of combination both AAA and AAB, with a similarity coefficient of 0.53-0.61; include Pisang Raja Jambe (AAA), Raja Kriyak (AAA), Raja Klutuk (AAB), Raja Brentel (AAB), Raja Seribu (AAB), and Raja Lini (AAB). Likewise, Cluster 3 also consists of combination both AAA and AAB, with a similarity coefficient of 0.43-0.58; include Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Bandung (AAB) and Raja Gareng (AAB). Surprisingly, Pisang Raja Bali (ABB) was positioned as an out-group with the lowest similarity coefficient of 0.22. It may due to the small amount of polymorphisms obtained from the amplification of several RAPD primers in Pisang Raja Bali sample. Whilst, Pisang Monyet (AAw) and Kluthuk Wulung (BBw), the wild relatives of banana cultivars (previously assumed to be the outgroups)

Table 4. Genome inference comparison of Pisang Raja based on morphology, ITS PCR-RFLP, Microsatellite and RAPD markers.

No.	Pisang Raja	Morphology*	ITS PCR-RFLP **	Microsatellite***	RAPD (this study)
1	Raja Bali	ABB	ABB	ABB	ABB
2	Raja Bandung	ABB	ABB	ABB	AAB
3	Raja Delima	AAA	-	-	AAA
4	Raja Kisto	AAA	AAB	-	AAA
5	Raja Gareng	AAB	-	-	AAB
6	Raja Jambe	AAA	AAA	-	AAA
7	Raja Kriyak	AAA	-	AAA	AAA
8	Raja Madu	AAA	AAA	-	AAB
9	Raja Sereh	AAB	-	AAB	AAB
10	Raja Seribu	AAB	AAB	AAB	AAB
11	Raja Kutuk	AAB	-	-	AAB
12	Raja Lini	AAB	AAB	-	AAB
13	Raja Brentel	ABB	ABB	-	AAB
14	Monyet	AAw	AAw	AAw	AAw
15	Kluthuk Wulung	BBw	BBw	BBw	BBw

* Reference genome based on morphology: Jumari & Pudjoarinto, 2000; Sukartini, 2007; Wahyuningtyas, 2009; Hapsari, 2014; Hapsari *et al*., 2015b, Nedha *et al*., 2017.

** Reference genome based on PCR-RFLP ITS: Ekasari *et al*., 2012; Hapsari *et al*., 2015a; Hapsari *et al*., 2018.

*** Reference genome based on Mikrosatelit: Wahyuningtyas *et al*., 2009; Retnoningsih *et al*., 2010.

were reveal nested in Cluster 2, with a similarity coefficient of 0.56.

PCo analysis was conducted in order to confirm the grouping pattern of Pisang Raja based on clustering analysis. Result of PCo scatters plot diagram was also grouped into 3 main clusters according to its genomes. The diagram was providing a clearer picture ordination of 13 Pisang Raja examined (Figure 3). The grouping patterns based on RAPD markers in this study was presumably caused by genetic differentiation among Pisang Raja cultivars. Some of the factors may cause genetic differentiation, such as geographical isolation and habitat fragmentation (external), as well as internal factors such as mutation, natural selection, genetic drift, and gene flow (Slatkin, 1987).

Genome inference comparison of Pisang Raja based on morphology, ITS PCR-RFLP, microsatellite and RAPD Markers.

Upon this study, RAPD markers are able to reveal the high genetic diversity among Pisang Raja from Java Island as indicated by a high level of polymorphisms. Nonetheless, the grouping pattern for the purpose of genome inference on some bananas was considered moderate in accuration and consistency. This study showed that several Pisang Raja were confirmed to have the same genome identity as the morphological results, such as Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Jambe (AAA), Raja Kriyak (AAA), Raja Gareng (AAB), Raja Sereh (AAB), Raja Kutuk (AAB), Raja Seribu (AAB), Raja Lini (AAB), and Raja Bali (ABB). However, some cultivars show different results, include Pisang Raja Bandung, Raja Madu, and Raja Brentel (Table 4).

In addition, genome inference from this study compared to other molecular methods such as ITS PCR-RFLP and microsatellite showed same results on some cultivars and also differed on the others (Table 4). For example, Pisang Raja Bali and Raja Madu were confirmed ABB and AAA respectively, according to all molecular methods. Pisang Raja Kriyak was confirmed AAA, both using RAPD and microsatelite. Pisang Raja Lini was confirmed AAA, both using RAPD and ITS PCR-RFLP. Whilst, Pisang Raja Kisto was inferred AAB according to ITS PCR-RFLP but AAA according to RAPD (Table 4).

Further, Pisang Raja Bandung in this study was identified as AAB genome, whereas according to morphological characterization and other molecular methods (ITS PCR-RFLP and microsatellite) was considered as ABB. Likewise, Pisang Raja Madu was considered as AAA by morphology and ITS PCR-RFLP, whereas in this study it was confirmed as AAB. Meanwhile, Pisang Raja Brentel according to morphological characterization by Nedha *et al.* (2017) upon sample from Kediri (East Java) was identified as ABB, whereas in this study upon a sample from Gunung Kidul (Central Java) was inferred as AAB. On the other hand, Hapsari *et al.* (2015b) concluded that Raja Brentel Warangan banana collected from the Yogyakarta region identified as AAB genome; and Raja Prentel (spelling variation) from Pasuruan (East

Java) identified as ABB genome.

The difference results in genome inference based on RAPD marker in this study was suspected because of the band absence on some Pisang Raja cultivars at certain primers (was not successfully amplified), thus the grouping pattern and the genome inference became less precise. This result was supported by the previous study by Sukartini (2008) which shows that some AAB cultivars were not clustered in one group, due to the small amount of polymorphism obtained from the amplification of each RAPD primer. In addition, the locality where was the material collected also presumably as a causing factor. Possibly, the material being studied was actually different. Indeed, the presence of numerous cultivar names and synonyms in different languages and dialects of the region become taxonomic problems. The same cultivars are known by different names in a different region. Occasionally, the same name is applied to distinct cultivars. Phonetic variations associated with tonal languages in Java often result to differences in spelling (Valmayor *et al*., 2000).

Each molecular method in predicting genomes of bananas has advantages and disadvantages, and also showed some inconsistency results. Genome inference using morphological approach has high subjectivity, morphological characters are influenced by environmental factors so that it influences the lack of result validity (Jumari & Pudjoarinto, 2000). The protocol of RAPD method is relatively simple, efficient, and will produce many fragments of DNA so that it can determine the level of polymorphism among organisms with good results. Further, RAPD method uses random primers which amplification processes may be initiated in several places and are dominant so that polymorphic DNA bands results are sometimes inconsistent, differing in band sizes and numbers. In addition, the use of RAPD markers can also provide different results if repeated (Demeke & Adams, 1994; Simpson, 2006; Poerba & Ahmad, 2013). Likewise, the microsatellite method has protocol similar to RAPD but uses specific primers that amplify only at one particular site, and are co-dominant with more informative and accurate results, however the lack of this method is that it requires high costs to design a new primer for specific organism (Wahyuningtyas *et al*., 2009; Retnoningsih *et al*., 2010). Meanwhile, ITS PCR-RFLP method uses even more specific marker, i.e. ITS region which provide more precise results of genome inference, but are technically more difficult because amplification results need to be incubated with specific restriction enzymes of *Rsa*I (Ekasari *et al*., 2012; Hapsari *et al*., 2015a; Hapsari *et al*., 2018).

Inference grouping of Pisang Raja genomes using RAPD markers in this study compared to other methods (morphology, ITS PCR-RFLP, microsatellite) was considered moderately accurate since some cultivars showed same results and also differed on the others. The genomic grouping in this study is the resulted of amplification of all RAPD primers which have not been specifically screened for primers which are suitable for banana cultivars (genome donor A) and (genome donor B). However, RAPD markers have several advantages that are relatively simple, fast, reliable, and quite accurate for checking polymorphisms and genetic variations of bananas to develop further policy on the breeding program and conservation (Pillay *et al*., 2000; Williams *et al*., 1990). The use of RAPD markers has some weakness in grouping the genomes of Pisang Raja (in this study), therefore further research with different techniques of more specifics markers is needed to get more specific and accurate results are necessary. Proposed other methods which more specific but complicated procedures are through ploidy analysis and nuclear DNA content with flow cytometry (Dolezel *et al*., 1997; Asif *et al*., 2001). In addition, there are other more specific molecular methods, i.e. trnL- F, rbcl, rflp, ITS

.

CONCLUSIONS

RAPD analysis of 13 Pisang Raja cultivars from Java Island showed high genetic diversity. About 12 out of 20 primers were successfully produced polymorphic bands. Polymorphism analysis showed that the 12 primers (OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20) were suitable and proposed to be used as markers for detecting genetic diversity in Pisang Raja, with OPA 2 and OPA 17 primers being the most effective primers. Pisang Raja cultivars examined were grouped into 3 main clusters following their genomes, namely AAA, AAB, and ABB at genetic similarity coefficient of 0.39. Molecular study of Pisang Raja using RAPD markers was able to describe the genetic diversity among Pisang Raja, however, the grouping pattern for the purpose of genome inference was considered moderate in accuracy and consistency. Therefore, further research using more specific genetic markers are needed to confirm the genome identity of Pisang Raja accurately.

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Research Article

Changes in Vegetation on Mount Agung Volcano Bali Indonesia

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ABSTRACT

Volcanic activity is a major natural disturbance that can catastrophically change an ecosystem over a short time scale. The eruption of Mt. Agung strato-volcano in 1963-1964 was considered among the most important volcanic event of the 20th century due to its effect on global climate. Studies on vegetation and landscape of Mt. Agung post-1970-1980 has been scarce. The current eruption of Mount Agung in June-July 2018, brought awareness of the importance urge to document the past and current landscape along with vegetation on Mt. Agung. Our study aimed to utilize remote sensing technique to explore the pattern of current (2017) land cover and vegetation density on Mt. Agung and estimate of vegetated areas and whether it has changed from the past. LANDSAT 8 images (www.earthexplorer.usgs.gov/) were used in this study. Supervised classification in ENVI was employed to obtain land use or land cover of the Mt. Agung area. Normalized Difference Vegetation Index (NDVI) was also calculated using the feature in the ARC GIS. Online web-based application, REMAP was used to obtain information on past and present condition of the crater of Mt. Agung to see whether there have been changes in vegetated areas around the crater using REMAP (www.remap-app.org). Results showed there are basically five main landcover that can be recognized namely forest (20758.23 ha), settlement (4058.37 ha), water area (41606.64 ha), open area (15335.64 ha) and farming (34554.78 ha). Our NDVI analysis also resulted in areas with have high density (78836.04 ha), medium density (15490.26 ha) and also no vegetation (31008.24 ha). Using web-based GIS application REMAP, we found that there has been an increase (approximately 1 km^2) in vegetation cover from the 1980s to 2016. The changes in vegetation near the crater of Mt. Agung is relatively slow when compared to another volcano such as Mt. Merapi. Remote sensing application has enabled us to obtain information on vegetation change relatively easily compared to conduct an extensive on-ground survey where more time and funding is needed.

INTRODUCTION

Volcanic activity is a major natural disturbance that can catastrophically change an ecosystem over a short time scale (2001). More than half of the active terrestrial volcanoes encircle the Pacific Ocean and are known as the 'ring of fire'. Indonesia is located on this chain of active volcanoes that stretched from west to east of the Archipelago (Sutomo 2013). With 130 active volcanoes lies in its region, Indonesia has become the most volcanic country on Earth (Weill 2004). One of the volcano in Indonesia which recently being on the center of attention is the Agung volcano in the Island of Bali. In September 29th, 2017 the volcano status was on the highest alert level due to its numerous volcanic activities. There has been debate and opinion that the volcano is surely set to erupt, then in 25th November 2017 Mt. Agung erupted.

The first ever recorded in history eruption of Mt. Agung was in 1843 (Dilmy 1965) and there is no complete report has been written following the eruption. The next catastrophic eruption was in 1963

-1964. The eruption of this strato-volcano in 1963-1964 is considered among the most important volcanic event of the 20th century due to its effect on global climate (Self and Rampino 2012). One year after 1963 of Mt. Agung eruption, almost 90% of the affected areas were still barren almost as if it had been cemented (Whitten *et al.* 1996). Few plant species that survive the eruption such as *Sambucus javanica*, *Eleusine indica,* and *Ageratum conyzoides* were found to be alive after a few months of the eruption (Dilmy 1965).

Change in land use and land cover has been a significant aspect of environmental management and conservation planning for many decades (Murray *et al.* 2017a). The role of remote sensing (RS) and geographical information systems (GIS) in ecology, especially in fire and vegetation management, has been recognized (Arno *et al.* 1977; Chuvieco and Congalton 1989; Keane *et al.* 2001; van Wilgen *et al.* 2000; Verlinden and Laamanen 2006). Van Etten (1998) used a GIS for predictive vegetation mapping using models that linked vegetation units to mapped environmental variables across the extensive remote areas of Hammersley Ranges in Australia.

Land cover maps permit the portrayal of the distribution of ecosystems and land cover types, assessments of biodiversity and identification of areas undergoing loss, fragmentation, and degradation (Haddad *et al.* 2015; Murray *et al.* 2017a). Studies on vegetation and landscape of Mt. Agung post 1970-1980 has been scarce. With the current eruption on Mount Agung in June-July 2018, it is of importance to document the past and current landscape along with is vegetation on Mt. Agung. Our study aimed to utilize remote sensing technique to explore the pattern of current (2017) land cover and vegetation density on Mt. Agung and estimate of vegetated areas and whether it has changed from the past.

Method

To obtain the current land cover and vegetation density on Mt. Agung and its surrounding, a satellite image for Mt. Agung (year 2017) was downloaded from LANDSAT 8 (www.earthexplorer.usgs.gov/). When selecting images to be download, we looked for images which were not covered by clouds or try to minimize the cloud cover percentage as much as possible with image quality level 9 (no errors detected, perfect scene). We then chose band 6, 5, and 3 and composite them into one image. After layer stacking, then cropping was done so that only Mt. Agung area was shown. This result then was load as RGB and used as the basis for classification. The classification was done using supervised classification, maximum likelihood approach with ENVI 4.5. Once classification finished, each class were converted to individual layer in a shapefile to be analyzed in ARCGIS 10.1.

We also use REMAP to obtain information on past and present condition of the crater of Mt. Agung to see whether there have been changes in vegetated areas around the crater. We use REMAP because it is difficult to find good past images from LANDSAT on Mt. Agung. Remap (https://remap-app.org) is an online mapping platform. Remap was developed to enable users to quickly map and report the status of ecosystems, contributing to a global effort to assess all ecosystems on Earth under the IUCN Red List of Ecosystems (Murray *et al.* 2017a). Remap uses the power of the Google Earth Engine, allowing users to directly access vast satellite data archives and state-of-the-art remote sensing methods. Remap handles the technical details of remote sensing so that users can focus on training, classifying and improving their maps (Murray *et al.* 2017b).

To obtain information on vegetation density, we used NDVI technique. NDVI is an index describing vegetation by showing the difference between near infrared (which is strongly reflected by vegetation) and red light (which is absorbed by vegetation). NDVI is correlated to vegetation biomass, vigour, and photosynthetic activity. This index exploits the reflectance patterns of ground elements in the red (R) and near-infrared (NIR) bands of the electromagnetic spectrum to distinguish green vegetation from its background soil brightness and is calculated as (NIR - R)/ (NIR + R). NDVI values range from -1 to 1, with positive values representing vegetated areas and negative values representing non-vegetated regions (Sankaran 2001). The NDVI ratio approach usually adopted for land cover change estimation in preference to the more commonly employed post-classification pixel-by-pixel comparison method (Lillesand *et al.* 2008) since it also permits the identification of areas where changes in the vegetative cover have been significant, but insufficient to cause change in class membership (Sankaran 2001). In our study, NDVI was generated using NDVI feature in ARC-MAP (ARC GIS 10.1) image analysis toolbar. Band 2, 3, 4, and 5 were chosen for Landsat 8 (OLI)image as input images in ARC-MAP which represent the blue, green, red and near-infrared (NIR) bands. By choosing image analysis tab, all the bands layers were composite into one and then the RGB channels were adjusted to just using only the NIR, red and green bands. Scientific output box was chosen on the NDVI tab in ARC-MAP so that instead of displaying the wavelength, it will give the value of +1 to -1 in the NDVI result. Once NDVI images

generated, colour scheme was applied for easier interpretation.

In addition, to obtain information regarding plant species that were occurred on Mt. Agung and its surrounding from past to present, we conducted a literature study into the database belongs to plant registration division of Bali Botanical Garden. The database holds the information on the past flora exploration (1970's) up to 2000 (Arinasa 2017).

Results and Discussion

Our result on landcover classification using Landsat 8 image and processed with ENVI is presented in figure 1. There are basically five main landcover that can be recognized namely forest (20758.23 ha), settlement (4058.37 ha), water area (41606.64 ha), open area (15335.64 ha) and farming (34554.78 ha). Our NDVI analysis also resulted in areas with have high density (78836.04 ha), medium density (15490.26 ha) and also no vegetation (31008.24 ha) (Figure 2). Most of the unvegetated areas located on the northern part of the mountain. This could indicate the direction of the eruption.

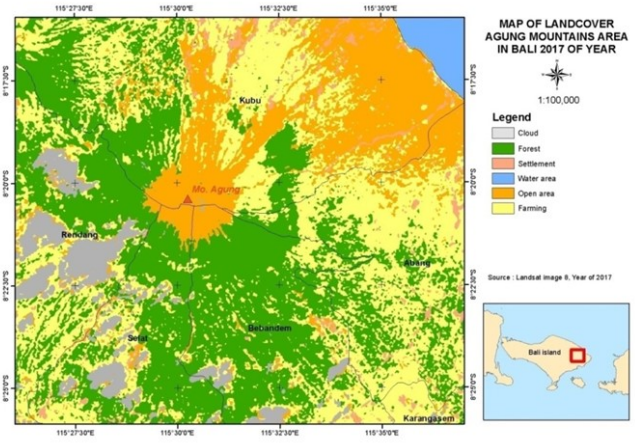

Figure 1. Landuse map of Mount Agung in Bali, using Landsat image 8 (2017).

Unlike eruption on Mt. Merapi, where the direction of pyroclastic flows mostly moved from the crater to the south flank of the mountain (Sutomo 2010), Mount Agung flows of eruption materials tends to move to the north. On 4th June 2006, the "*Geger boyo*" flank in Kaliadem (Sleman District, Yogyakarta Province) collapsed and *nuées ardentes* occurred until 14th June. The flows moved down the slope through Gendol River (Kaliadem area) and destroyed all vegetation and buildings in its path (Sutomo 2010). Offcourse the Agung Mountain has a different type of eruption with Merapi. Merapi type eruption is unique where it usually in a form of a pyroclastic flows or *nuees ardentes* that originated from a collapsed lava dome at the summit (Bardintzeff 1984), whereas Agung is more of an explosive volcanic type of eruption. The February 1963 to January 1964 eruption of Gunung Agung, Indonesia's largest and most devastating eruption of the twentieth century, was a multi-phase explosive and effusive event that produced both basaltic andesite tephra and andesite lava (Self and Rampino 2012). Perhaps due to this direction of eruption and lava deposit, we can see from the produced map (Figure 2), the northern part of the mountain are mostly seen as no vegetation or medium density vegetation. However, these results could also mean that on the northern part of the Island are probably populated by human which dwell up until along the north coastline.

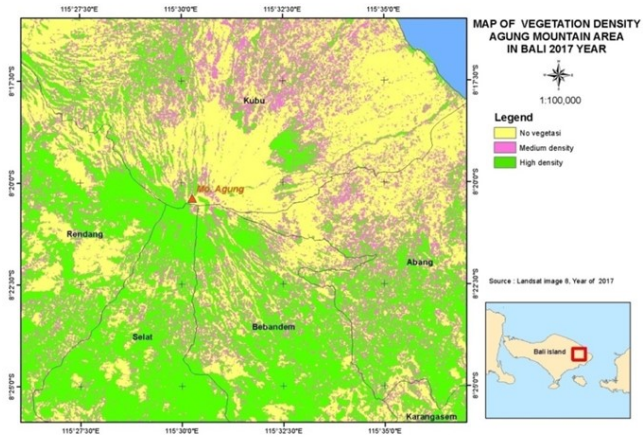

Figure 2. Vegetation density on Mount Agung and surrounding, using Landsat image 8 (2017).

Another approach to see the changes in vegetation cover following the eruption is to focus on the area surrounding the crater of Mt. Agung. Therefore, using web-based GIS application REMAP, we found that there has been an increase (approximately 1 km^2) in vegetation cover from the 1980s to 2016 (Figure 3). Remote sensing has also been applied to study vegetation succession on Mount Merapi. Yuniasih (2017) used NDVI approach to compare vegetation density in two locations of affected by 2010 eruption of Mt. Merapi and one that was not affected by the eruption. The study found that the location that was affected by pyroclastic flows of Merapi eruption have almost similar NDVI with location which was not affected, indicating the existence of the successional process.

It can be inferred from the results (Figure 3) that the rate of vegetation succession on Mt. Agung is slow compared with Mt. Merapi. In the first decade of primary succession, plant re-colonization on Mt. Merapi *nuées ardentes* deposits was rapid, with fifty-six species belonging to 26 families recorded. The highest number of species belonged to the Asteraceae, then Poaceae, followed by Fabaceae and Rubiaceae. The number of species presents varied as

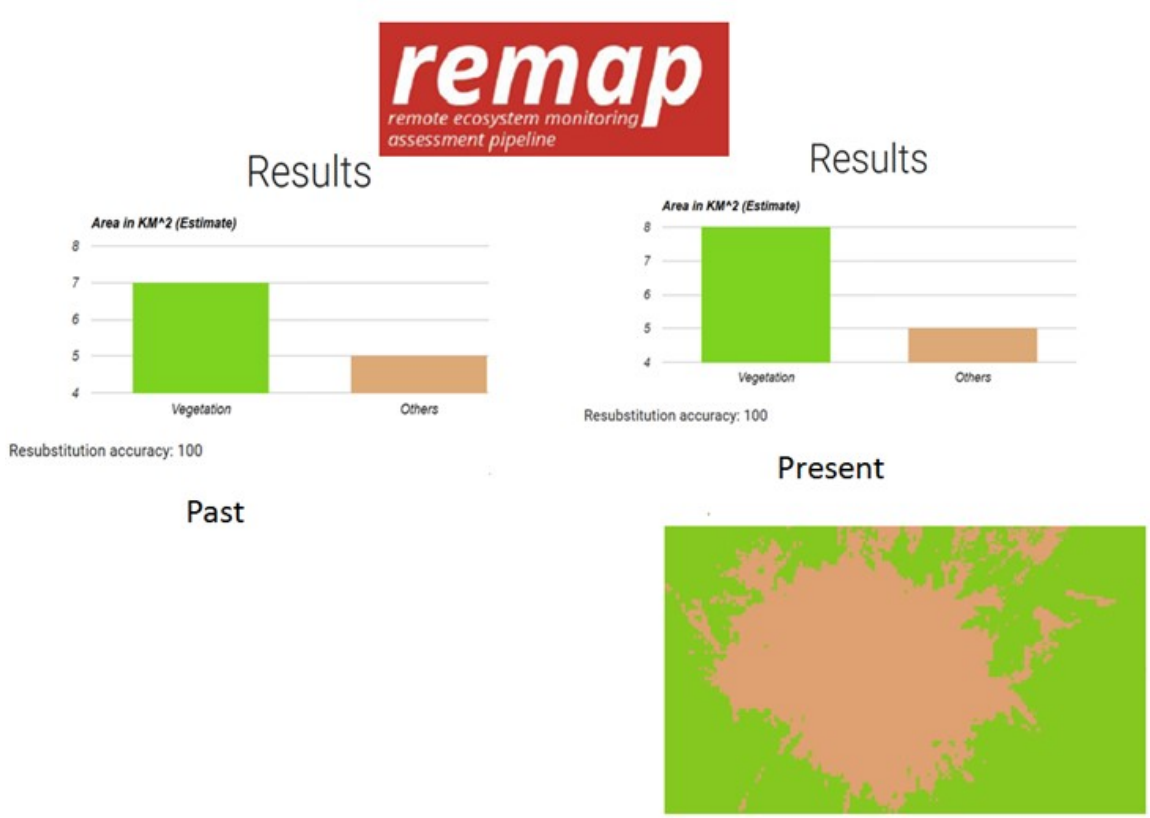

Figure 3. Vegetated areas changes on the surrounding summit (near the crater) of Mount Agung Bali using remap. Past refers to 1980's and present refers to 2016.

the deposit aged, with a rising trend of species richness and diversity over time (Sutomo *et al.* 2011). Unfortunately, studies on vegetation of Mt. Agung post 1970-1980 has been scarce. However, Dilmy (1965), reported that a few months after the 1963 eruption three species of plants were found in the Besakih vicinity on the slope of Mt. Agung namely *Sambucus javanica, Eleusine indica,* and *Ageratum conyzoides*, while all other plants were dead. Antos and Zobel (2005) report that the majority of types of herbs can penetrate to deposits of 4.5 cm or less, but at a depth of more than 15 cm most layers of herbs will die and cannot penetrate. One year later in 1964, Dilmy reported that there were 83 species consists of grasses, herbs, shrubs, and trees were found growing at the elevation of 900 to 1250 m above sea level. Pioneer tree species that Dilmy (1965) found one-year fater the eruption among others were *Albizzia procera, Albizzia montana, Engelhardia spicata, Ficus benjamina, Ficusseptica, Ficus ampelas,* and *Melia azedarach.*

According to Dilmy (1965), these plants were found along dikes and water courses in moist places. This highlight the importance of microsites or safe sites which facilitate pioneer plants to grow. There has been abundance research on safe sites and their importance for seedling recruitment and establishment on a disturbed areas (Eriksson and Ehrlén 1992; Jumpponen *et al.* 1999; Moral and Wood 1993; Tsuyuzaki *et al.* 1997). Sutomo and Hasanbahri (2008) studied pine species (*Pinus merkusii)* recovery in Kaliadem forest of Mt. Merapi which was affected by the 2006 eruption. Needles and stem branches of the pine that fall on the surface of the sediment serves as mulch and helps provide it nutrients needed for pine seedlings to grow. In addition, the morphology of the rocky deposits protects pine seedlings from herbivory by animals. Thus recovery of Pinus in Kaliadem forests already have a good source of seedlings 'capital'.

Another approach that we can use to obtain a description of what plant species constitute on the Mt. Agung areas is by studying expedition records from a nearby botanical garden. "Eka Karya" Bali Botanical Garden-Indonesian Institute of Sciences (LIPI) as a plant conservation unit has conducted series of plant expedition from the 1970s up to 2000s to explore plants species including general taxa and also specific such as an orchid. A summary of the results is displayed in table 1. Among these results, there are seven species that similar to the species that were reported by Dilmy one year after the 1963 eruption namely *Pteridium aquilinum*, *Musa* sp., *Clerodendron serratum*, *Homalomena* sp., *Hibiscus rosa -sinensis*, *Vernoneaarborea*, and *Litsea* sp.

The duration of a succession process will depend on many things but among them is how severe the damage is and how much area is affected, whether there is a biological legacy (such as the source of seeds/location in the location and

Table 1. List of plant species collected from exploration (1970 – 2000) by Bali Botanical Garden on Mount Agung and its surrounding

Collection year	Species name	Family	Location
1970s	*Eria multiflora*	Orchidaceae	Bebandem village
	Phalaenopsis sp.	Orchidaceae	Tihingan, bebandem, karangasem
	Dendrobium sp.	Orchidaceae	Abang village
	Aegle marmelos	Rutaceae	Tista village, Abang
	Acriopsis sp.	Orchidaceae	Batugunung
	Piper sp.	Piperaceae	Batugunung
	Arachnis sp.	Leguminosae	Batugunung
	Vanilla sp.	Orchidaceae	Batugunung
	Musaenda sp.	Rubiaceae	Batugunung
	Opuntia sp.	Cactaceae	Kubu, Karangsem
	Euphorbia sp.	Euph.	Kubu, Karangsem
	Bulbophyllumbiflorum	Orchidaceae	Karangasem
	Dendrobium plicatile	Orchidaceae	Lempuyang Hill
	Appendicula angustifolia	Orchidaceae	Lempuyang Hill
	Dendrobium sp.	Orchidaceae	Lempuyang Hill
	Corimborchis sp.	Orchidaceae	Lempuyang Hill
	Phaius sp.	Orchidaceae	Lempuyang Hill
	Thevetia peruviana	Apoc.	Lempuyang Hill
1980s	*Santalum album*	Santalaceae	BatuGiling, Kubu, Karangasem
	Kalanchoe sp.	Crass.	BatuDewaKubu, Karangasem
	Lygodium sp.	Lygodiaceae	BatuDewa, Kubu, Karangasem
	Clerodendron sp.	Verb.	BatuDewa, Kubu, Karangasem
	Acacia cincinnata.	Leguminosae	abang
	Acacia polystachyaBenth.	Leguminosae	abang
	Caladium sp.	Araceae	abang
	Euchrestahorsfieldii	Leguminosae	abang
	Garcinia dulcis	Clusiaceae	abang
	Begonia sp.	Begoniaceae	abang
	Raphodopora sp.	Araceae	abang
1990s	*Ardisia humilis*	Primulaceae	Gunung Agung Forest
	Syzygium racemosum	Myrtaceae	Gunung Agung Fores
	Phaius tankervilleae	Orchidaceae	Gunung Agung Forest
	Calanthe veratrifolia	Orchidaceae	Gunung Agung Forest
	Goodyera sp.	Orchidaceae	Gunung Agung Forest
	Pandanus tectorius	Pandanaceae	Gunung Agung Forest
	Coelogyne flexousa	Orchidaceae	Gunung Agung Forest
	Dodonaea sp.	Sapindaceae	Gunung Agung Forest
	Pteridium sp.	Pteridaceae	Gunung Agung Forest
	Musa sp.	Musaceae	Gunung Agung Forest
	Platea sp.	Icac.	Gunung Agung Forest
	Dianella sp.	Xanthorrhoeaceae	Gunung Agung Forest
	Orthosiphon aristatus	Lamiaceae	Gunung Agung Forest
	Clematis sp.	Ranunculaceae	Gunung Agung Forest
	Nephrolepis duffii	Nephrolepidaceae	Gunung Agung Forest
	Clerodendron serratum.	Verbenaceae	Gunung Agung Forest
	Asplenium caudatum	Aspleniaceae	Gunung Agung Forest

Table 1. Cont'd.

Collection year	Species name	Family	Location
1990s	*Weinmannia blumei*	Cunoniaceae	Gunung Agung Forest
	Vanda tricolor	Orchidaceae	Gunung Agung Forest
	Dendrobium sagitatum	Orchidaceae	Gunung Agung Forest
	Hippeastrum sp.	Amaryllidaceae	Gunung Agung Forest
	Dendrobium linearifolium	Orchidaceae	Gunung Agung Forest
	Homalomena sp.	Araceae	Gunung Agung Forest
	Anaphalis sp.	Compositae	Gunung Agung Forest
	Phreatia secunda	Orchidaceae	Gunung Agung Forest
	Magnolia champaca	Magnoliaceae	Gunung Agung Forest
	Laplacea amboinensisMiq.	Theaceae	Gunung Agung Forest
	Platea sp.	Icac.	Gunung Agung Forest
2000s	*Mesuaferea*	Clusiaceae	Dsn. Brahma
	Hibiscus sp.	Malvaceae	Dsn. Brahma
	Cajanus cajan	Leguminosae	Dsn. Brahma
	Delichos lablab	Leguminosae	Dsn. Brahma
	Zingiber pupureum	Zingiberaceae	Dsn. Brahma
	Coleus amboinensis	Lamiaceae	Dsn. Brahma
	Michelia sp.	Magnoliaceae	Dsn. Dukuh
	Arenga sp.	Arecaceae	Dsn. Dukuh
	Curcuma sp.	Zingiberaceae	Dsn. Dukuh
	Garcinia mangostana	Clusiaceae	Dsn. Dukuh
	Musa sp.	Musaceae	Dsn. Dukuh
	Parmentiera sp.	Bignoniaceae	Dsn. Dukuh
	Alpiniagalanga .	Zingiberaceae	Dsn. Dukuh
	Curcuma sp.	Zingiberaceae	Dsn. Dukuh
	Zingiberofficinale	Zingiberaceae	Dsn. Dukuh
	Musa paradisiaca	Musaceae	Dsn. Dukuh
	Mangifera caesia	Anac.	Dsn. Dukuh
	Cocos nucifera	Arecaceae	Dsn. Dukuh
	Gmelina arborea	Verb.	Pempatan village, Karangasem
	Gmelina arborea	Verb.	Pempatan village, Karangasem
	Litsea sp.	Laur.	Lebah village, Rendang, Karangasem
	Meliosma sp.	Sab.	Munduk village, Karangasem
	Homalomena sp.	Araceae	Munduk village Karangasem
	Calanthe veratrifolia	Orchid.	Munduk village Karangasem
	Saurauia sp.	Saurauiac.	Munduk village Karangasem
	Ligustrum glomeratum	Anac.	Munduk village, Karangasem
	Trevesia sundaica	Anac.	Munduk village, Karangasem
	Vernonia arborea	Aster.	Munduk village, Karangasem
	Begonia longifolia	Beg.	Munduk village, Karangasem

Source: Plant registration division, Bali Botanical Garden-Indonesian Institute of Sciences (LIPI)

surrounding location) and the presence or absence of ecological intervention. Ecological intervention is human intervention to accelerate the natural succession process. This is called ecosystem restoration. In addition to ecosystem restoration efforts, it is also necessary to monitor or monitor ecosystem dynamics, especially the dynamics of plant vegetation in the volcanic region. Remote sensing technology can be used to monitor vegetation in the volcano area. Satellite image data in different years can be collected and processed for analysis and comparison on whether there is a change in the area of vegetation, whether there is a change in vegetation density or is there a change in the greenness index of vegetation or there may be changes in land use from the vegetation area to the area for other purposes. Given the level of damage and change of land in lowland forests mainly on Java

and Bali, it is now undeniable that mountainous/ upland forest areas (including volcanoes) play a very important role and become a place where high biodiversity can still be we find.

Although from the results there has been an improvement in terms of vegetated areas and also increase in species richness over time, however, as the threat of habitat and ecosystem destruction due to the consequences of climate change and anthropogenic disturbance increases, these volcanic-forests highlight the continuing need and importance of research on plant community succession and restoration on a volcanic terrain in Indonesia.

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Research Article

Variability and Intra-Specific Classification of Lima Bean (*Phaseolus lunatus* L.) from Timor Island based on Morphological Characters

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ABSTRACT
Lima bean (*Phaseolus lunatus* L.) is a species of beans which originating from the regions of Central America and Andes Mountains. Lima bean in Timor Island is underutilized although these plant growth there and have many variations. This study aims to determine the diversity of lima beans on Timor Island based on morphological characters. Samples were collected by survey methods from three districts on the island of Timor. Morphological traits related to the vegetative and flowering stages and mature seeds morphology were scored using the International Plant Genetic Resources Institute lima bean descriptors with a soft modification. The similarity index is calculated using the General Similarity Coefficient Gower formula. The dendrogram is generated from cluster analysis using the *Unweighted Pair Group Methods using Arithmetic Average* (UPGMA) method. Furthermore, *Principal Component Analysis* (PCA) was used to determine the role of each morphological character used. The dendrogram shows that 23 collected accessions are divided into two main clusters with a 57% similarity index. The two clusters are distinguished based on the presence or absence of secondary colors and secondary patterns in the seed organs. Then, each main cluster is divided into two subclasses based on the character of the pigmentation stem, the length of the terminal leaflets, flower color, and seed type.

INTRODUCTION
Phaseolus lunatus that known as lima bean is one of the important seeds that belonging to the family Fabaceae. This plant is one of the important legume plants which contain essential acids. It is one of the underutilized legume groups, but its nutritional content can be used as an alternative to overcome the malnutrition problem for people in developing countries (Kyerematen, 2015; Arora, 2014). This plant have many vernacular names based on dispersed in many country, such as *butter bean, sieva bean, madagascar bean* (En), *Haricot de lima, pois du Cap, pois souche, pois savon* (Fr), *Feijao de lima, feijao favona, feijao espandinho* (Portugal), *Mfiwi* (Sw), *sibatse simaron, patáni, zabache* (Philipina), *thua rachamat* (Thailand); *dâu ngu* (Vietnam). In Indonesia, lima bean has a lot of local names, such as *kacang mas, roay* (Sunda), *kara, kratok* (Java), *kratok, gribig* (Madura), *saru* (Minahasa), *merah bean* (Pontianak), and *koto, arbila* (Timor) (Baudoin, 1989).

Lima bean has a good adaptation capability in the tropics especially in less fertile, high humidity soils, dry climate and wet soil PH (Yaguiu, 2003; Kole, 2014). The geographic distribution of lima bean is widespread from arid climate region to high humidity areas. Lima bean is living in lowlands to highlands (50 – 2750 m asl) approximately (Bauodin *et al.*, 2004). The distribution area of lima bean in the world is Mexico, Guatemala, Ecuador, Peru, Colombia, Madagascar, Spain, parts of Africa, spread to Asian regions, especially the Philippines, Indonesia (Java), Myanmar (Burma) and Mauritius. In the late 19th century, this plant was brought and cultivated in Europe and Asia, initiated from the Philippines to Myanmar, then to Java Island. Lima bean began to be cultivated in the Minahasa region

and around Java. In addition to the area, in Bali and East Nusa Tenggara, these seeds are consumed by mixing with staple foods, namely rice, corn and cassava (Baudoin, 2006; Smykal *et al*., 2015).

Lima bean is the annual to perennial climbing plants which have a hood shape of standard flowers and twinning keels. The wild lima bean species show uncertain climbing growth habits, with prolonged flowering periods and large pod production (Zoro Bi *et al*., 2003). This type of pole usually shows a twining feature with a large and perennial rootstock. There is also a form of annual shrub developed in cultivation (Santos *et al*., 2008). The seeds have a rich variant, consist of the shape, size, color, and eye appearance. Sometimes the color of pale green flowers is purple (Beyra & Artiles, 2004) and its size, is smaller than common bean (*Phaseolus vulgaris*).

Botanical varieties of lima bean consist of *var*. silvester for the wild material and *var*. lunatus for the domesticated one. Furthermore, there are three major gene pools: One Andean gene pool (A) and two Mesoamerican gene pools (MI and MII); with one domestication event in each of them: 1) for A, the midaltitude western valleys between Ecuador and Peru in South America; 2) the central eastern region from Mexico for MI; and 3) the region located between Guatemala and Costa Rica for MII. Within the var. lunatus, Baudet (1977) indicated the existence of three cultigroups: 1) Sieva, with medium -sized and flat seeds; 2) Potato, with small globular seeds; and 3) Big lima, with large flat seeds. Big Lima represents the A gene pool, while Sieva and Potato represent the MI and MII gene pools (Silva, *et al*., 2017).

In Indonesia, varieties of lima bean consist of four groups. They are 1) java bean, have red seed and contains HCN; 2) red Rangoon/Burma bean, have small red seed with white spot and not contain HCN; 3) white Rangoon/Burma bean, have small white seed and not contain HCN; and 4) kratok/ lima bean, white flat seed and not contain HCN (PROSEA, 1993). Besides that, Purwanti & Prihanta (2017) divide lima bean from East Java into two main cultigroups based on classification according to (Baudet 1977). There are 1) medium-large size cultigroup (Sieva-Big lima group) and 2) small-medium sized seed (Potato-sieve group).

Lima bean in Timor Island has many seed shapes and colors. But the information and utilization of this plant in East Nusa Tenggara is very limited (Koten *et al*., 2013; Mundita, 2013). This greatly affects the conservation and breeding efforts of a plant. Plant breeding can be started from the selection of genetic diversity in a crop species, selecting the best variety for cross mains, to create cultivars that have high production, disease resistance, with vigorous growth (Purnomo *et al*., 2015). Therefore, the collection, evaluation and characterization and to show their diversity in genetic, morphological, or physiological is necessary. Morphological characters are important basic data in expressing genetic diversity. Besides that, the morphological character may be used to identify germplasm collections duplication, genetic diversity estimation study, and correlational study between morphology and other important agronomical traits (Purwanti & Prihanta; 2017). The objective of this research was to determine the diversity of lima bean based on morphological characters. The study is important to identify the range of variation and morphological similarity among accession using cluster analysis. The result is useful for the identification of characters selection on lima bean cultivation and conservation.

MATERIALS AND METHODS

Materials

This study was conducted in March 2016 to October 2016. Twenty-three sample accession was obtained from 19 villages in Kupang District, Timor Tengah Selatan District, and Timor Tengah Utara District (Table 1). The sampling locations have altitude ranging from 235 m asl – 1250 m asl.

Methods

Sample or accession collection was conducted based on observation and plant survey method. A sample of lima beans are stem, leaves, flowers, fruits, and seeds. The morphological data were taken in the field directly and make their specimen vouchers and take picture for determination. Morphological characterization is done by coding and scoring based on Biodiversity International (International Board for Plant Genetic Resources, Rome, Italy; IBPGR, 1982) with soft modification (Table 2).

Morphological data were analyzed by description for characterization to construct identification key. Similarity index was counted by Gower General Similarity Coefficient formula based on morphological characters data. Cluster analysis was conducted by UPGMA (Unweighted Pair Group Methods using Arithmetic averages) analysis to create a dendrogram with MVSP (Multivariate Statistical Program) v.3.1 software. Principal component analysis (PCA) was also performed to define the role of each morphological character in the grouping of accessions.

Table 1. Plant materials.

No.	Accession code	Location (Village/District)	Local name	Morphological Characteristics
1	TU1	Oerinbesi/Timor Tengah Utara	Koto fui	Flower purple; seed red purplis, plain, , small type
2	TU2	Manusasi, Timor Tengah Utara	Koto	Flower purple, seed brown with stripe pattern and white second color, medium-big type
3	TU3	Eban, Timor Tengah Utara	Koto	Flower white, seed maroon without pattern and second color, small-medium type
4	TU4	Benpasi, Timor Tengah Utara	Koto fui	Flower purple, seed black without pattern and second color, medium-big type
5	TU5	Lapeom, Timor Tengah Utara	Koto	Flower purple, seed white with spot pattern and red second color, small-medium type
6	TU6	Fafinesu, Timor Tengah Utara	Koto molo	Flower white, seed yellow without pattern and second color, big type
7	TU7	Fafinesu A, Timor Tengah Utara	Koto	Flower purple, seed purple-reddish without pattern and second color, small-medium type
8	TS1	Maunum, Timor Tengah Selatan	Koto fui	Purple Flowe purpler, seed purple-redish without pattern and second color, small-medium type
9	TS2	Supul, Timor Tengah Selatan	Koto bibi	Flower white, seed white, maroon second color, big type
10	TS3	Nobi-Nobi, Timor Tengah Selatan	Kot'molo	White Flower white, seed yellow without pattern and second color, medium-big type
11	TS4	Mnelalete, Timor Tengah Selatan	Koto	Flower purple, seed black without pattern and second color, small-medium type
12	TS5	Binaus, Timor Tengah Selatan	Koto fui	Flower white, seed purple-reddish without pattern and second color, small-medium type
13	TS6	Binaus, Timor Tengah Selatan	Koto fui	Flower purple, seed black without pattern and second color, medium-big type
14	TS7	Binaus, Timor Tengah Selatan	Koto	Purple Flower purple, seed brown without pattern and second color, medium-big type
15	TS8	Binaus, Timor Tengah Selatan	Kot'bibi	White Flower white, seed white with spot pattern and maroon second color, medium-big type
16	TS9	Benlutu, Timor Tengah Selatan	Koto	Flower purple, black seed without pattern and second color, medium-big type
17	TS10	Tuppan, Timor Tengah Selatan	Koto	White Flower white, seed purple-reddish without pattern and second color, medium-big type
18	KP1	Oben, Kupang	Arbila	Flower purple, seed white with dot pattern and maroon second color, medium-big type
19	KP2	Teunbaun, Kupang	Arbila	Purple Flower purple , seed black with dot/line pattern and white second color, medium-big type
20	KP3	Besmarak, Kupang	Arbila	White Flower white, seed white with dot/line pattern and second maroon color, medium-big type
21	KP4	Oeletsala, Kupang	Arbila	White Flower white, seed white with dot pattern and red second color, medium-big type
22	KP5	Takari, Kupang	Arbila	Flower purple, seed white with dot pattern and red second color, medium-big type
23	KP6	Camplong II	Arbila hutan	White Flower white, seed black without pattern and second color, small-medium type

RESULTS AND DISCUSSION

Morphological variability

Based on the observation, morphology variation of lima bean are very striking on leaf, fruit flowers and seeds. Lima bean has oval to round of leaf shapes (Figure 1. B1 & B2). It is determined by the ratio of the length and width of the leaf terminals. In the flower organ, the variation appears in the flag and wing part. The flag has a two-color variation, green and green-purple. The colors of the wing part have two variations, white and purple (Figure 1. C1 & C2).

In part of fruit organs variation appears in pods curve, there are straight and slightly curved pod shape (Fig. 1-D). Seed variation appears on their

Table 2. Characters and Character states of morphological character of lima bean based on IBPGRI (1982) with modifications.

No.	Characters	Code	Character state
1	Growth habit	H	(1) *deterrminate*; (2) *indeterminate semiclimbing* (3) *indeterminate climbing*
2	Differences of main stem and branches	PWBTM	(1) yes; (2) no
3	Pigmentation of main stem	PBU	(0) no; (3) on nodus; (5) widespread
4	Leaflet lenght	PND	(3) 5-7 cm; (5) 9-11 cm; (7) 13-15 cm
5	Leaflet shape	BD	(1) bulat < 1,5; (3) oval 1,5-2; (5) oval-lanset 2-3; (7) lanset 3-6
6	Margin main leaf	KTLD	(0) no; (3) limited ; (5) widespread
7	Leaf color	WD	(3) light green; (5) dark green
8	Color of flower keel	WLA	(1) green; (2) pink to purple
9	Color of flower standard	WSA	(1) white-greenish; (3) purple-greenish
10	Color of flower wings	WSB	(1) white; (3) light pink; (5) lilac
11	Wing opening	PS	(0) parallel wings-closed; (3) intermediate opening; (5) wings widely diverging
12	Raceme position	PoPB	(3) within foliage; (5) intermediate; (7) emerging from leaf canopy
13	Pod beak shape	BPP	1) short beak; 2) medium length beak; 3) long beak; 4) thick beak
14	Position of pod bearing racemes	PPB	(1) Mainly concentrated at the base; (2) Mainly concentrated in the middle; (3) Mainly concentrated at the top; (4) Evenly distributed throughout the plant; (5) Variably distributed
15	Orientation of pod bearing racemes	OPB	(1) perpendicular; (2) linear
16	Cross section shape of pods	PMP	(1) flat; (2) round-cone; (3) ellipse; (4) number 8 shape
17	Pod curvature	LP	(1) straight; (2) slightly curved; (3) curved
18	Distribution pod on stem	DPB	(1) variably distributed; (2) mainly concentrated at base ; (3) mainly concentrated at the top
19	Number seed per pod	JBI	(1) 1 – 2; (2) 3 - 4 ; (3) > 5
20	Seed shape	BBI	(1) round; (2) *oval-cuboid*; (3) kidney shape
21	Seed primer color	WDBI	(1) white; (2) gray; (3) yellow; (4) brown ; (5) maroon; (6) purple-reddish; (7) black
22	Seed second color	WKBI	(0) no color; (1) white; (2) light brown/orange; (3) dark brown; (4) red; (5) purple-reddish
23	Second pattern color	PWKBI	0) no pattern; (1) line; (2) specks; (3) blotches; (4) mixture
24	Seed coat pattern	PBWKBI	0) no pattern; 1) pattern around eye only; 2)eye distinct with few specks on body; 3) eye with blotches on body; 4) eye linked to other parts of pattern, blotches and some specks may be present; 5) eye not clear with soft specks
25	Hilum shape	BHI	(1) round; (2) elips; (3) oval; (4) lancet
26	Textur of testa	TT	(3) Smooth; (5) Moderately ridged; (7) Markedly ridged
27	Seed length (cm)	PBI	(1) < 1; (2) 1 - 1,5; (3) > 1,5
28	Seed width (cm)	LBI	(1) < 0,8; (2) 0,8 - 1,3; (3) > 1,3
29	Seed thickness (cm)	TBI	(1) < 0,4; (2) > 0,4

shape, size, color, and color pattern of the seed skin (Fig.2). Based on the size of the length, width, and thickness of the seeds the types of seed consist of four types of seeds. They are *big type* is the accession that belongs to the large seed group, the medium type that dominates in this grouping. It is divide into *medium-big type* and *small-medium type* and the last *small type*. This classification is made by Yaguiu *et al.* (2003) and Asante *et al.* (2008), that grouping seeds into five types of seeds based on seed size (length, width).

The color of seeds and the color pattern of lima seeds showed a very high variation. In his research, the color that dominates is a brownish-yellow color and the second color is dark brown. The results of this study showed that lima seed shells have a primer color of seeds and a second color that forms a certain pattern. The primer color of the seeds varies widely are white, yellow, brown, maroon, purple-reddish, and black. The second color on the body of the beans that form the color pattern are white, dark brown and red. Color patterns formed in the form of lines, dots, stains/ spots, and mixtures. Based on the morphological characteristics of the color and the color pattern that associated with the seed hilum areas in seed terminology, lima bean are grouped into three groups, (1) Plain seed (Fig. 2. A), seeds have one color only without any color patterns; (2) color with hilum seed (Fig. 2. B-1,2,3), seeds have hilum eye is

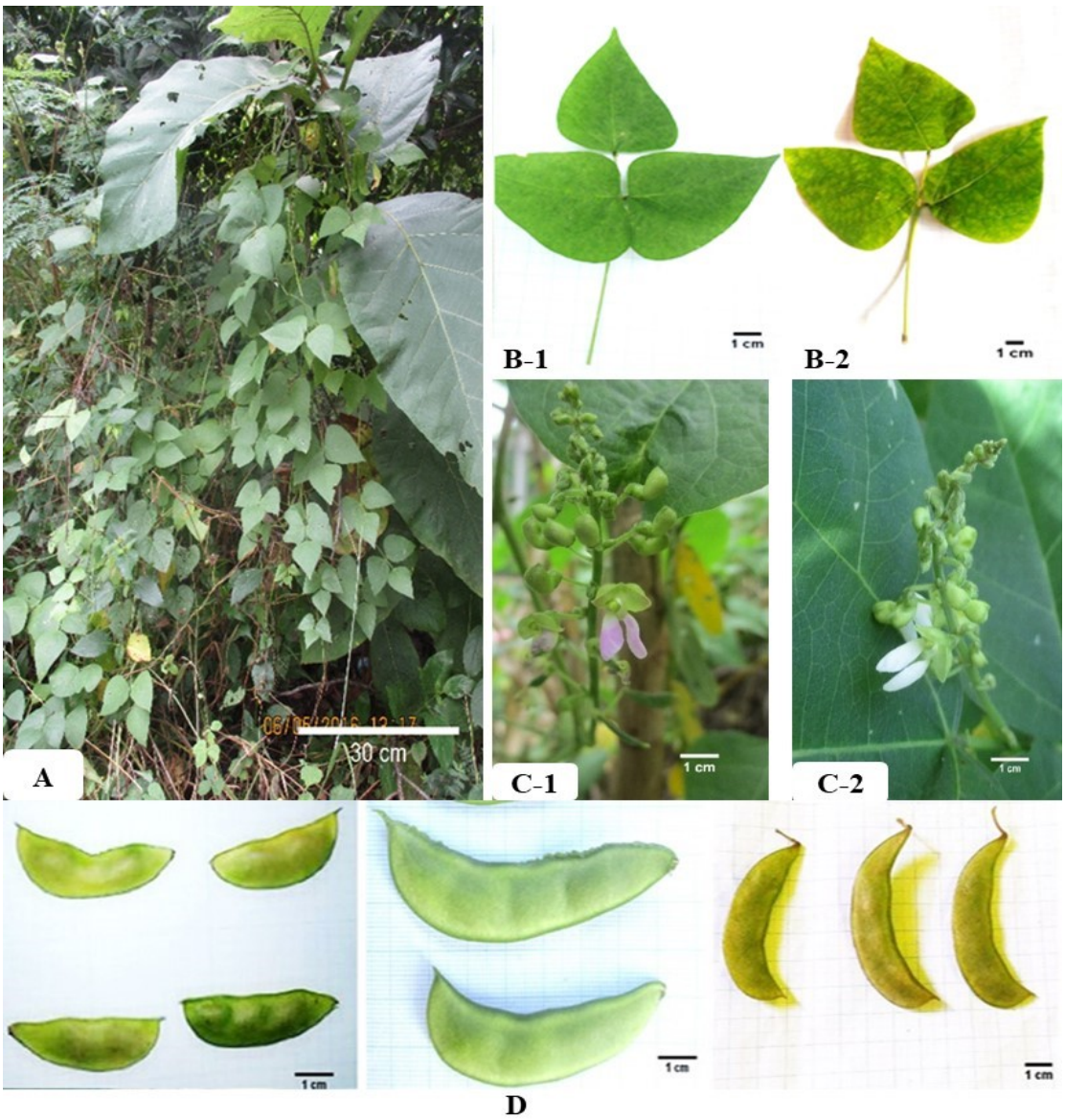

Figure 1. Morphological variation of lima bean. A. habit; B. Leaflet shape (B-1: oval, B-2: rounded); C. Color of flowers (C-1 purple flower, C-2 white flower); D. variation of pod shape.

associated with other pattern parts in the presence of spots and points on the seed body; (3) color with unclear hilum (Fig. 2. B-4,5), seeds have unclear eyes with spots on the seed body.

Cluster ana;ysis and principal component analysis

The combination of cluster analysis and principal component analysis has a purpose to result in a fundamental empirical role of morphological characters in grouping accession (Sari *et al.*, 2016). The result of cluster analysis showed two main clusters on the dendrogram (Figure 3) with a similarity coefficient of 0.57 - 1. Both of this cluster divided by secondary seed color. Cluster A has the secondary color of the seed and cluster B is devoid of the secondary color of the seed. Each group accession has a combination of morphological characters which is a marker of each accession group known as a distinguishing character that can be seen in Table 3.

Cluster A is divided into two subgroup groups A1 and A2. The distinguishing characteristics of these two subgroups are main stem pigmentation, wing opening, flower color, length and width of the seed. This character can be seen in Table 4. Martinez -Casstillo *et al.* (2004) classified this group as the cultivation group with papa-sieva seed (intermediate type). Cluster B is divided into subgroups B1 and B2. Distinguishing characters between these two subgroups are the terminal leaf terminals, flower color, and seed length. This character can be seen in Table 5.

Based on its origins, Debouck (1994) reveals that the white lima bean flowers are the Central American type and the purplish color is the Andes type. Furthermore, in relation to domestication, Martinez-Castillo *et al.* (2003) in his research revealed that the purple flowering variant is a type of cultivation similar to the wild type and the white

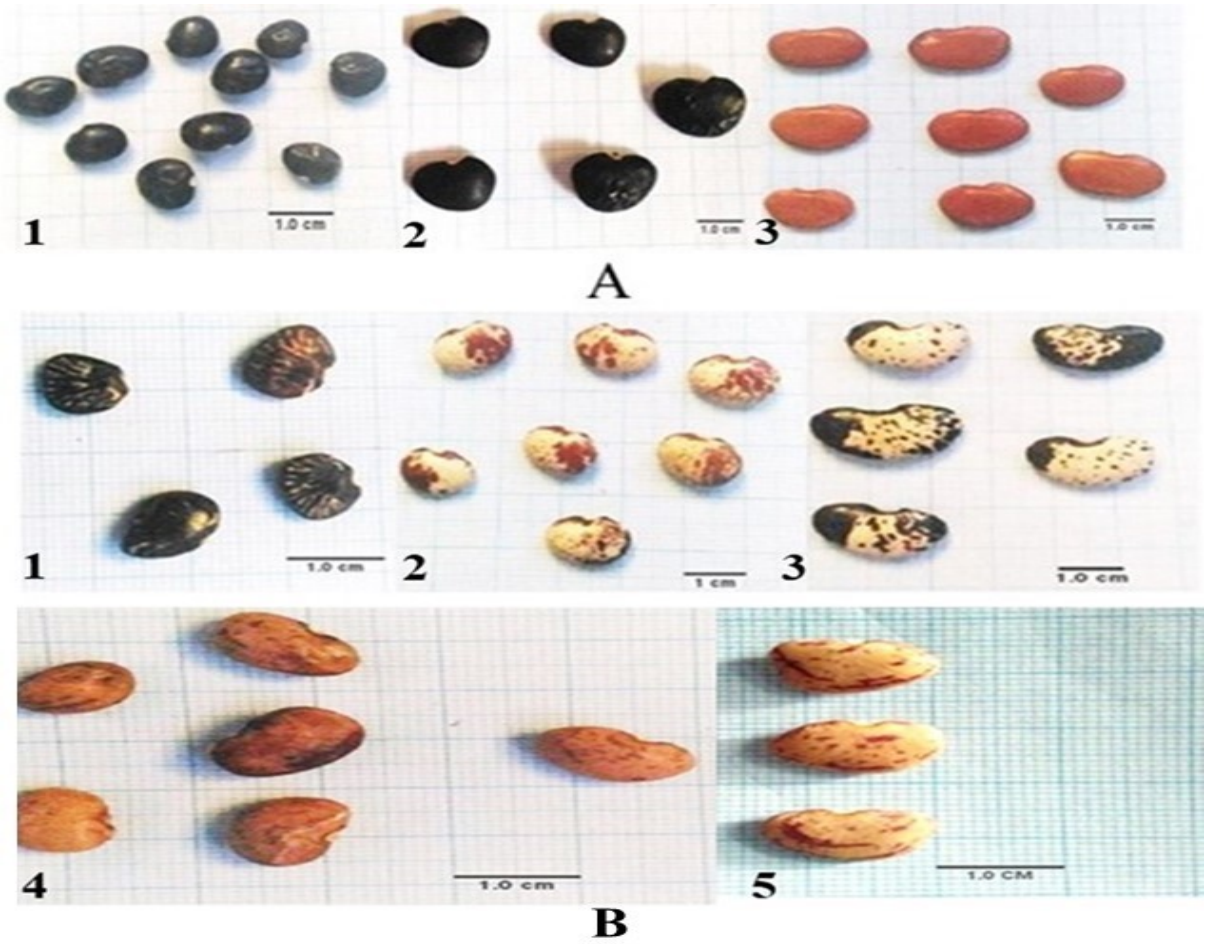

Figure 2. Variation of seed shape and color. (A) Seed without secondary color and pattern, (B) Seed with secondary color and pattern.

flowering is the type of cultivation. Kole (2014) stated that almost all cultivated crops of lima beans have white flower color also. This occurs because of the gene flow between wild-type germ plasma and cultivation type at some time ago. This statement can also be observed in the field. Generally, the white flowering is a variant of the most widely cultivated by local farmers.

Lioi (1994) states that lima bean has a high polymorphism especially on seed character. The size of lima bean seeds found is 0.9 - 2.5 cm long, 0.8 to 1.4 cm wide and 0.1 to 1 cm thick. Bauodin *et al.* (2004) revealed that local varieties of Central America are characterized by small seed groups. Local varieties of South America are characterized by large seed groups. It was stated by CSSA (2010) that the big seed varieties were domesticated in the Andean mountains, while the medium and small varieties originated from the western part of Central Mexico. However, both small seeds and large seeds are found in South America but not in the same habitat. In addition to seed size, lima bean has a variety of seed shape also. The form of seeds in this study was oval-cuboid and kidney form. This result was also presented by Martinez-Castillo *et al.* (2004) in collecting wild-type samples, local varieties and cultivated varieties in the Yucatan Peninsula, Mexico. The shape of round-elliptical seeds, grouped into papa groups and whose form of kidneys are grouped into sieva groups.

Yaguiu *et al.* (2003) argued that the basic color of lima beans is black, red, brown, white, yellow, and brownish brown generally. The pattern of color on the skin of the seeds, among others, specks, only on the hilum, dotted, and strike. Furthermore, Montero-Rojas *et al.* (2013) in his study revealed the existence of other basic colors other than those proposed by Yaguiu *et al.* (2003) is the color purple. According to Gepts (2012), the color produced on the skin of the seed is a biochemical pigmentation process which is a flavonoid compound and includes anthocyanin. The production of pigments in stems, flowers, and fruits is a characteristic or wild type character of all species of cultivation. The pattern of the skin color of lima beans is closely related to the color of the stems, flowers, and fruits. However, the range of development of pigment and its expression on the skin of seed is larger than other organs. In this study, no color variation found in leaf organ like any other organ. This indicates that in leaf organs there is no proanthocyanin which has the ability to produce different colors. This is also stated by Onyilagha &

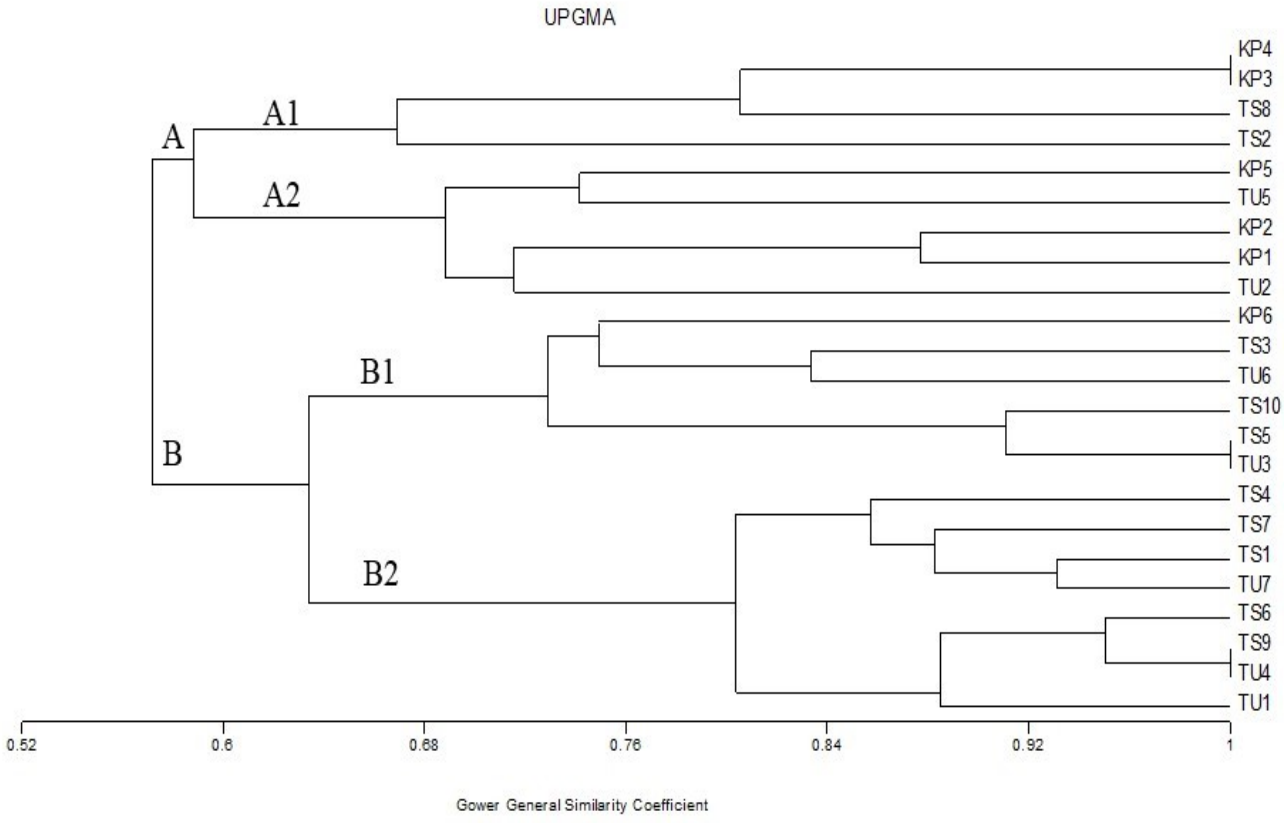

Figure 3. Dendrogram of 23 accession lima bean based on morphological characters used UPGMA analysis.

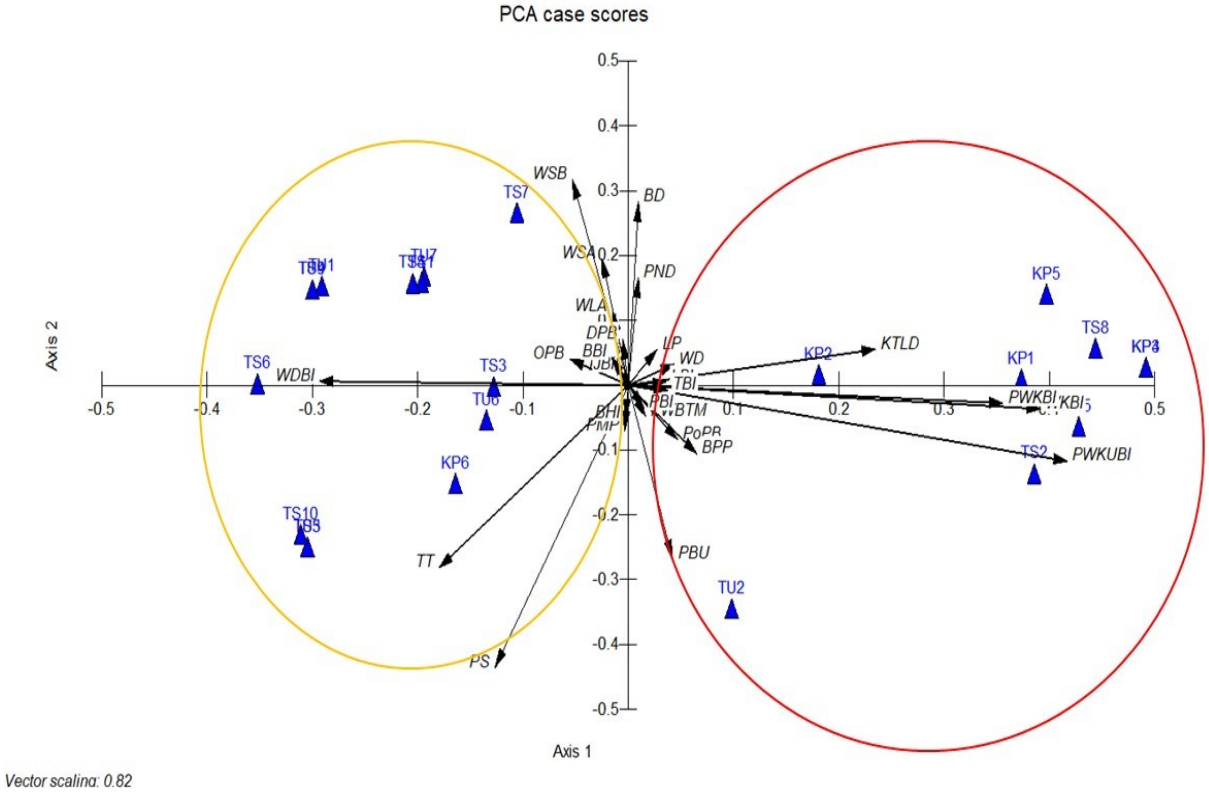

Figure 4. Diagram pattern of grouping accession and spread pattern of morphological characters of *Phaseolus lunatus* that define grouping accession.

Table 3. Plain and color patterned seed distinguishing characters.

No.	Character	Group A	Group B
1	Basic color of seeds	White, brown, black	Yellow, brown, purple, black
2	Secondary color of seeds	White, red	None
3	Second color pattern of seeds	lines, dots, stains / spots, and mixtures	None
4	Seed coat color pattern	The eye is related to other parts of the seed	None

Table 4. Distinguishing Character of Subgroup A1 and A2.

No.	Character	Group A-1	Group A-2
1	Stem pigmentation	None	Diverse
2	Wing opening direction	Parallel	Parallel, intermediate
3	Flower color	White	Purple
4	Seed type	medium – big	small – medium

Table 5. Distinguishing character of subgroup B1 and B2.

No.	Character	Group B – 1	Group B – 2
1	Length of terminal leaflet	5 – 7 cm	9 – 11 cm
2	Flower color	White	Purple
3	Seed type	medium – big	small–big

Islam (2009) who studied the compounds of flavonoids and other phenol in *Phaseolus* group that is cultivated.

Gepts (2014) states that domestication of *Phaseolus* resulted in several changes, such as reduced seed dormancy, seed dispersion, photoperiod, and increased variety of pod and seed shape and color. This is also supported by research results Martinez-Castillo *et al.* (2004), where the wild type has plant organs of varying color and shape is relatively small. In addition to this wild type, there are also intermediate types. The type still has the same characteristics as the wild type, but the variations are quite large and can be consumed. This is thought to be the result of gene flow between wild-type germ plasma and cultivation type at some time ago. It is also thought to be the result of a regressive mutation of a process seen in various tropical environments.
The distribution of the cluster on the dendrogram does not indicate a clear grouping between accessions by geographic area. This led to the grouping in this study not to be recognized formally as subspecies (Sari, *et al.*2016). Therefore, the morphological approach in this study supports the status of the research object as a species with high phenotype variability but is not divided into taxa that represent the formal category under the species based on the concept of the taxon. The role of each character in forming clusters on cluster analysis can be seen in the main component analysis (Figure 4).

The results of the main component analysis in the two-dimensional plot are not only showing the direction but also showing the small and large roles of each characters, which show as vectors with different length (Susandarini, *et al.*, 2013). In this research, the eigenvalue value which is seen to show the important role in grouping is the eigenvalue which the magnitude is ≥ 0.3. The accession group formed in the first axis consists of two groups A and B. The most important distinctive characters are the base color of the seed, the secondary color of the seed, the secondary color pattern and the color pattern of the two seed shells. Santos *et al.* (2010) suggest that it is this character first used to recognize the main gene pool and also to characterize the genetic diversity of the species.

Information obtained on morphological variations based on cluster analysis and major component analysis can be used to reveal the intraspecies classification of lima bean. The result of cluster analysis in the form of phenetic kinship between accessions is used as the basis for the classification of intraspecies based on open classification or informal classification (Martinez-Castillo *et al.*, 2004; Purnomo *et al.*, 2015). Based on morphological characters which distinguishing lima bean accession, the informal classification of *P. lunatus* in Timor Island divided into two group accession, that is plain seed and pattern seed. Grouping of 23 accession *P. lunatus* in Timor Island shown in Table 6.

Based on the combination of morphological characters that distinguish *P. lunatus* in Timor Island, the parallel type of key for identification taxa of *P. lunatus* :

1. a. have a secondary color on the seeds, and have a color pattern ……..…… 2
   b. does not have a secondary color and is not patterned on the seeds ……..……...3
2. a. there is no pigmentation in the stem, white flower, the type of sieve-big seed…..……A-1 (color seed-white flower group)
   b. diverse stem pigmentation, the color of the purple flower, the type of potato-sieve seed…….A-2 (color seed-purple flower group)
3. a. length of leaflets terminal 5-7 cm, white flower color, type medium – big seed…….B-1 (plain seed-white flower group)
   b. length of leaflets terminal 9 - 11 cm, purple flower, type small – medium seed……B-2 (plain seed-purple flower group)

Table 6. Grouping of intra-specific 23 accessions *P. lunatus*.

Group	Accession code
Plain seed lima bean	KP1, KP2, KP3, KP4, TS2, TS8, TU2, TU5
Pattern seed lima bean	KP5, KP6, TS1, TS3, TS4, TS5, TS6, TS7, TS9, TS10, TU1, TU3, TU4, TU6, TU7

CONCLUSIONS

Twenty-three accessions of lima beans (*P. lunatus*) in Timor Island divided into two main clusters: (1) pattern seed and (2) plain seed lima bean group. Pattern seed group that have secondary and patterned colors are grouped into two subgroups based on stem pigmentation, flower color, and seed type. Plain seed group that does not have secondary and non-patterned colors in seeds are grouped into two subgroups based on terminal leaf length, flower color, and seed type. This classification could be informally classified into two cultivated varieties, namely poisonous and edible varieties.

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Research Article

The Antidepressant Effect of *Chlorella vulgaris* on Female Wistar Rats (*Rattus norvegicus* Berkenhout, 1769) with Chronic Unpredictable Mild Stress Treatment

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ABSTRACT
Depression is a disabling mental disorder, predicted to become the world's number 2 disability by 2020 by the World Health Organization (WHO, 2018). Chronic stress is one of the triggers for depression, causing an imbalance in brain chemicals and antioxidants levels. Although antidepressant is a common treatment, discomforting side effects has compromised its efficacy, prompting the search for alternative medicines. *Chlorella vulgaris* is a microalgae famous for its excellent protein and antioxidant content. In this study, *C. vulgaris* (360 mg/kg p.o.) potency of antidepressant in chronic unpredictable mild stress (CUMS) model of depression in female rats was evaluated compared to amitriptyline (2,25 mg/kg p.o.) for 14 days. Two types of *C. vulgaris* namely cultivation sourced and commercially-sold, were used. Sucrose preference test, forced swim test (FST) and open field test (OFT) were used as depression-like behaviour test to validate *C. vulgaris* effect. Adrenal glands were observed to further understand its effect on the stress organ. The CUMS method produced rats with depressive-like behaviour evidently by reduced body weight, sucrose preference, exploring behaviour in OFT, and increased immobility duration in FST. Furthermore, an increase in adrenal weight, fasciculata zone, and reticularis zone was observed. Both *C. vulgaris* significantly (p<0,05) reversed depressive-like behaviour in rats subjected to CUMS, but not the size of adrenal glands. This finding indicated both types of *C. vulgaris* has the potential to be an alternative antidepressant but because of the short duration of treatment, it's speculated that *C. vulgaris* may not have exhibited enough difference structurally yet.

INTRODUCTION
In modern times, depression has become a disability that is common in Indonesian society. Research by the Ministry of Health in 2018 states that 6.1% of people in Indonesia are depressed. Unfortunately, from all recorded patients, only 9% were treated by professionals (Ministry of Health, 2018). One of the reasons being depression treatment which is still limited to antidepressant drugs that often bring uncomfortable side effects to the body. The common side effects include heart palpitations, insomnia, agitation, headache, and even in extreme cases, stroke (Hedaya, 2011).

One of the alleged cause of depression is chronic stress (Hammen, 2005). Chronic stress can cause a decrease in serotonin and noradrenaline (Natarajan *et al.*, 2015; Goddard *et al.*, 2009). It can also lead to oxidative stress which causes low levels of antioxidants in the body (Srivastava & Kumar, 2015). In the human body, stress is responded by the adrenal gland. The medulla adrenal will response through the sympatho-adrenomedullary pathway while the adrenal cortex response through cortisol production from the fasciculatory zone via the hypothalamus-pituitary-adrenal axis. Cortisol belongs to the glucocorticoid group which increases blood sugar levels in the body for the provision of instant energy. This energy is used to fight or flee from the stressor (fight or flight response) (Chung *et al.*, 2011). Stress response process generates reactive

oxygen species (ROS) as a byproduct. A high ROS concentration in the body can result in various poor health conditions, one of them is lipid peroxidation in brain which inhibits the binding of serotonin (Scapagnini, *et al*., 2012). ROS effects can be reduced by antioxidant.

Serotonin is synthesized from the amino acid tryptophan, while noradrenaline uses the amino acid tyrosine (Hemat, 2004). Both of these amino acids can be obtained from food, one of which is microalgae such as *Spirulina* sp. and *Chlorella* sp. (Santhanam, 2015; Gershwin & Belay, 2008). According to Bewicke & Potter (2009), *Chlorella vulgaris* contains tryptophan and tyrosine in high content. In addition, *C. vulgaris* is also superior in chlorophyll content. Chlorophyll has been studied to have excellent antioxidant activity (Hsu *et al*., 2013) so that the high chlorophyll in *C. vulgaris* can give an insight of its potential as a source of antioxidants for humans.

Chlorella vulgaris has long been used as health supplements for humans and are safe for consumption (Klamczynska & Mooney, 2017). However, it is not yet popular, because of the expensive price due to complicated commercial processing, which is the process of removing cell walls from *Chlorella* to make it easier to digest (Zheng *et al*., 2011). Production with traditional cultivation methods omits said process, providing a *Chlorella* alternative at a more affordable price, but there is no research to determine the difference in performance between the two yet. Seeing the potential for the discovery of alternative medicine to reduce depression from a natural, affordable source, we conducted this study. Two types of *Chlorella vulgaris* were used (cultivated and commercially sold) to further determine the differences that may exist between the two.

MATERIALS AND METHODS

Materials

Twenty-five female Wistar rats (R. *norvegicus)* aged 1-2 months with an interval bodyweight of 100-150 grams were used as animal models and were obtained from Laboratory of Advanced Research and Testing Unit IV, University Gadjah Mada, Yogyakarta. *C. vulgaris* powdered extract was obtained from Blue Green Microalga Technology. Commercially sold *C. vulgaris* tablets were obtained from CNI Sun Chlorella. The antidepressant drug used as a control was amitriptyline (Indofarma). Supporting materials include sucrose powder, ice cubes, distilled water (CV Progo Mulyo), and wood shavings bedding. Animal feed was given in the form of AD II pellets and *reserve osmosis* (RO) water was given as drinking water, both *ad libitum*, meaning the animals can access these freely. Animal models were kept in groups in a cage with each cage containing 5 rats. Rice husk as standard bedding was changed every 2-3 days. Ketamine (Ketalar): xylazine (Interchemie) cocktail with ratio 1: 1 was used as an anaesthetic and euthanasian agent. Materials needed for making histological slides include neutral buffered formalin (NBF) 10% (CV Progo Mulyo), graded ethanol (CV Progo Mulyo), physiological salt solution (0.9% NaCl), Ehrlich Hematoxilin dye, Eosin Y 1%, distilled water, xylol, toluol, paraffin temperature 57-60^{o}C, Meyer's albumin, object-glass, and cover glass.

Animal models

Animal models were adapted in cages and research environment. Acclimation was carried out for 3 days to minimize stress in animals before the research was conducted so the data has good validity. This stage was carried out in the research room II Laboratory of Advanced Research and Testing Unit IV, University Gadjah Mada. Animal models were weighed once a week individually using semi-analytic scales. The weighing was also done before blood sampling for the determination of the anaesthetic dose and before euthanizing for the calculation of the adrenosomatic index.

Blood sampling was carried out for blood glucose level measurement at the beginning of the study after the acclimation process, after stress induction and after the treatment period. Before sampling, animals were anaesthetized first with ketamine (50 mg/kg BW). Blood sampling was done by inserting one end of the micro hematocrit inward *medial canthus* of the animal's eye, (forming a 30-degree angle to the nose) and was then stored in 1 mL microtube. Blood glucose measurements were made with a glucose meter (EasyTouch) with corresponding singe use test strips.

Stress Induction by Chronic Unpredictable Mild Stress (CUMS) Method

Animal models stress was induced by the method CUMS (Hu *et al*., 2017; Zhang *et al*., 2014). The stressors given were described as follows:

a. Treatment of cold water, animal models were put in a water tank with a water depth of 25 cm and a water temperature of 5^{o}C for 3 minutes. After treatment, animals were allowed to dry themselves inside a dry cage with fresh wood-shavings bedding while illuminated with a lamp to keep the animals warm.
b. Treatment of warm water, animals were put in a water tank with a water depth of 25 cm and a water temperature of 45^{o} C for 3 minutes. After

treatment for this stressors is the same as cold water treatment.

c. Treatment of wet cages, animals were placed in cages with wet wood shavings bedding as high as 5 cm. This treatment was given for 24 hours. After treatment, the wet bedding was removed and replaced with dry, standard bedding.
d. Dark-light cycle reversal, animals were exposed to light at night (18.00-06.00) and in a dark place during the daytime (06.00-18.00).
e. Sound wave exposure, animals were exposed to the ultrasonic sound for 12 hours.
f. Treatment of tilted cage, animal's cages were tilted 45^{o} for 24 hours.
g. Food and water deprivation, for 24 hours animals were not fed and the water bottles were removed from the cage. The bedding was replaced beforehand to remove food burrowed in it by the animals. After treatment, feeding was continued normally and water bottles were given back.

These stressors were carried out to all groups except control for 42 days, by giving 1 type of stressor per day. The order of stressors was maintained so that the same stressors did not occur for 2 consecutive days. On day 42, animals underwent behavioural tests to determine their stress state.

Sucrose Preference Test (SPT)

The test was carried out at the beginning of the study, after stress induction and after the treatment period. The test was started by habituation of animals to two drinking bottles for 3 days. After 3 days, one of the bottles was filled with 2% sucrose solution and the other was filled with RO water. Animal cages were then not equipped with drinking bottles for 12 hours, after which both bottles were provided together. The position of the bottle was exchanged every 12 hours and the consumption of sucrose solution and RO water was measured after 24 hours. Sucrose preference was then calculated based on the following formula: Sucrose preference (%) = (Sucrose solution consumption / Total water and solution consumption) mL x 100% (Shukkoor *et al.,* 2016)

Open Field Test (OFT)

The test was done after stress induction and after the treatment period and refers to the method used by Shukkoor *et al.* (2016) with changes. Animals were placed in the arena of the lidless opaque plastic box (30 cm x 60 cm with height 40 cm) without bedding individually for 10 minutes. The movements and behaviours of the animals were recorded using a camera during this period. After 10 minutes, the animal was removed from the arena.

Forced Swim Test

This test was done immediately after OFT was done. This protocol follows the literature from Slattery & Cryan (2012). This test consists of two sessions separated by a 24 hour period. At each session, animals were placed in a water tank made of clear acrylic (diameter 20 cm and height 50 cm), which was then filled with water at 26^{o}C. Water filled the cylinder to a depth of 25 cm so that the entire body of the animal can be submerged and to prevent the animal's front limbs from reaching the edge of the container and running away.

The day before the test, 15 minutes of the pretest was carried out to habituate animals to the test environment. The next day, the animal was individually put on a water tank to swim for 5 minutes and immobility time was observed. The animal was then transferred to a dry cage containing fresh bedding and illuminated by the lamp as warmer. After the animal's body was relatively dry, it was returned to their respective cages. Faecal boli that may be present in the water tank were removed, and water was replaced every 3 test or if necessary.

Per oral Treatment

This study was designed randomly with 5 treatment groups. The treatment group consisted of 1 control group, 1 stress control group, 1 positive control group treated with 2,25mg/kg BW Amitriptyline and 2 groups of variations in treatment, namely treatment of cultivated *C. vulgaris* and treatment of commercial *C. vulgaris,* both given at 360mg/kg BW dosage. Each group consists of 5 replications. The duration of treatment is 14 days after stress induction. Treatment was given every morning between 9:00 and 11:00.

Euthanasia and surgery

On day 56 (or after the treatment period), the animals were terminated by the exsanguination method. Adrenal glands were cleaned and used to find out the adrenosomatic index according to the formula: adrenosomatic index = (total organ weight / total body weight) gram x 100% (Avinashe, 2013). Adrenal glands were then moved to neutral buffer phosphate (NBF) 10% solution to fixate it. Then the histological preparations were made with Hematoxylin-Eosin staining.

Data Analysis

Obtained data were analysed using one-way ANOVA with a confidence level of 95% (α <0,05) and Duncan Multiple Range Test as post-hoc test. Statistical analysis was done with SPSS 23 program and graphs needed were made using Microsoft

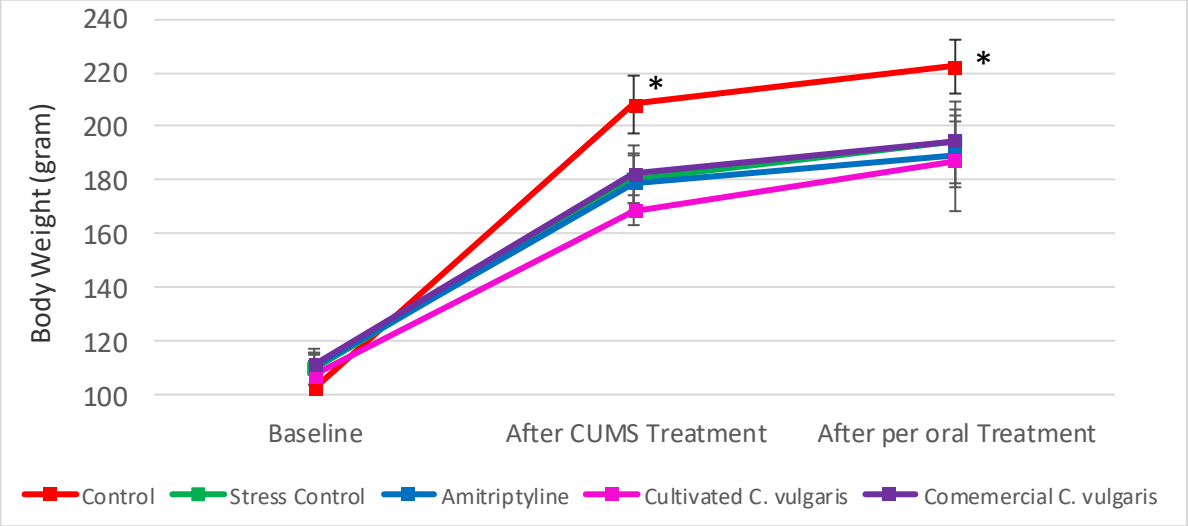


Figure 1. Weight gain in female Wistar rats during the study. * $p < 0.01$, when compared with CUMS, treated groups; one-way ANOVA followed by Duncan *post-hoc* test.

Excel. Qualitative data in the form of roaming videos were analysed using idTracker software (created by Perez-Escudero *et al.* (2014) and can be downloaded free via the web http:// www.idtracker.es /) to see trajectory patterns made by the animal during the test period.

RESULTS AND DISCUSSION

This study has passed the required ethical feasibility test by the Animal Ethics Committee of Universitas Gadjah Mada before the study was conducted with a certificate number: 00042/04/ LPPT/VI/2018

Body Weight

Bodyweight is data that can be linked to animal physiological conditions. In this study, animal body weight was weighed at the beginning of the study, after CUMS treatment and after *per oral* treatment. Changes that might occur were observed.

Figure 1 showed that all group experienced weight gain from the beginning of the study until the end of CUMS treatment, with control group having a significant difference ($F=8.504$; $df=4.18$; $p< 0.01$) compared to all other groups. This can be caused by stress in CUMS treated groups which caused the tendency to consume fewer amounts of food (hypophagia) (Rabasa & Dickson, 2016). Stress will cause the body to release various stress hormones like CRH, ACTH, and cortisol which in turn will prepare the body for the "flight or fight" condition. This condition includes increased blood pressure, gluconeogenesis rate, and blood supply to the muscles, heart, and brain. Therefore, organ activities that require energy but not related to this response such as digestive and reproductive organs will be temporarily inhibited (Yau & Potenza, 2014). *Per oral* treatment did not cause a difference afterwards compared to the stress control group (Figure 1). This could be due to the short period of treatment which did not provide enough time for structural change to develop. Furthermore, the adaptation to stressors in the form of increased calorie use efficiency in the stress control group (Rabasa & Dickson, 2016) and late age factor could also play a role in means of a slower metabolism, which could explain the minimum weight gain observed (Barnett, 2017).

Blood Glucose Levels

Blood glucose level can describe stress status in rat. Figure 2 showed that the stress treatment given did not have a significant effect on blood glucose.

During the initial period of the study until stress conditions were reached, all groups experienced elevated blood glucose levels with insignificant differences. After going through *per oral* treatment according to the research design, the control and stress control group had a decreased blood glucose value whereas the remaining treatment group experienced an increase.

In theory, stress can increase glucose levels acutely through the work of the epinephrine and cortisol hormones which stimulate liver glycogenolysis and gluconeogenesis. These processes will return to normal conditions (homoeostasis) after the stressor ends (Preiser, 2016) so that an increase in blood glucose is also possible but when the measurement was done, the animal has been able to restore its blood glucose level to its initial state.

Besides that, it was found that microalgae treatments can increase blood glucose levels, seen from the elevated value that happened after *per oral*

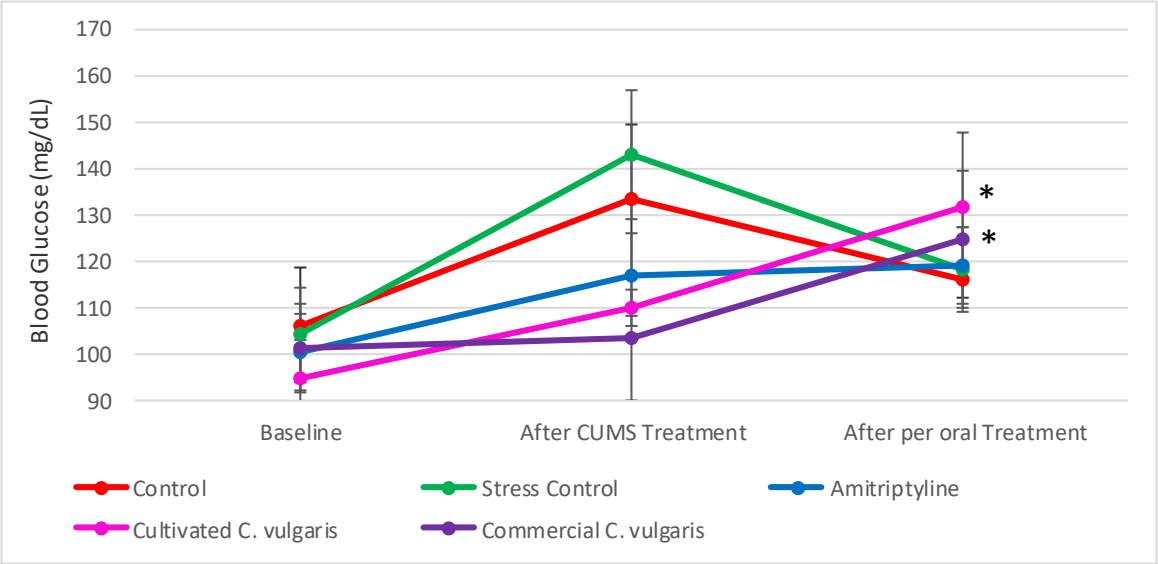


Figure 2. Blood glucose level of female Wistar rats at the beginning of the study, after CUMS treatment and after *per oral* treatment. * $p<0.05$ when compared to its respective group on its previous period (Baseline and after CUMS treatment).

period (F=10.829; df=2.12; $p<0.05$). According to Safi *et al.* (2014), the carbohydrate content of *C. vulgaris* is quite high, at around 55% of its dry weight, with the predominance of carbohydrates in the form of sugar as a constituent of its cell walls like galactose, glucose, xylose, arabinose, and mannose. Cultivated *C. vulgaris* provide a higher increase than commercial *C. vulgaris* because cultivated *C. vulgaris* are still intact with the cell wall, whereas in the commercial one which has been factory processed, this cell wall has been removed.

Sucrose Preference

The preference of animals against sucrose was measured using the sucrose preference test (SPT). Low preference reflects anhedonia behaviour, while high preference is generated by model animals under normal conditions.

On Figure 3, it can be seen that the sucrose preference of the control group stayed relatively high, unlike the other four groups which experienced a decline. This shows the anhedonia behaviour on groups with CUMS treatment which is one of the signs of depression.

After going through per oral treatment, the sucrose preference of animal in the treatment group of both amitriptyline and microalgae return to its original state and approached sucrose preferences of the control group. The animal in the stress control group had its sucrose preference decreased and differed significantly from other groups and even that group itself in the previous week (F=7.33; df=4.18; $p<0.01$), indicating that sucrose preference in this group continues to decline. Rats naturally have a tendency to consume sweet drinks, so that a decrease in sucrose preference can be assumed as anhedonic behaviour (Serchov *et al.*, 2016).

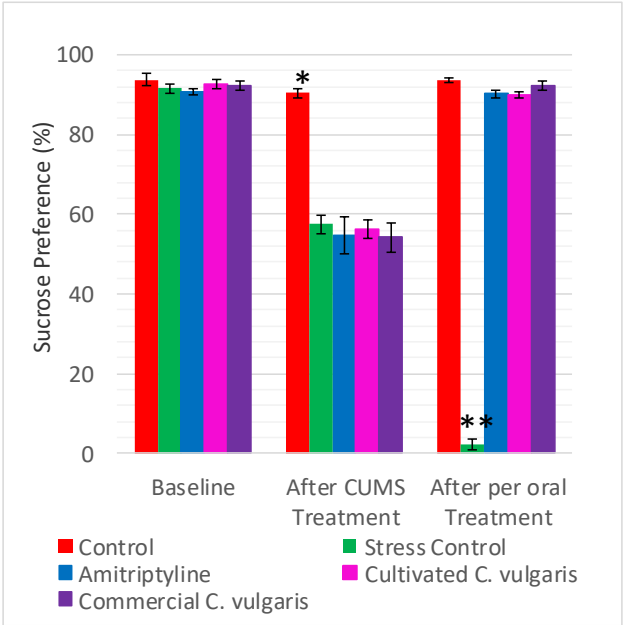


Figure 3. Sucrose preference of female Wistar rats at the beginning of the study, after CUMS treatment and after *per oral* treatment. * $p < 0.01$ compared with CUMS treated groups; ** $p<0.01$ compared with control and per oral groups; one-way ANOVA followed by Duncan *post-hoc* test.

Amitriptyline was chosen because of its efficiency compared to other types of antidepressant drugs in treating depression (Cipriani *et al.*, 2018). Amitriptyline works by increasing the concentration of serotonin and norepinephrine neurotransmitters at synapses (Gupta *et al.*, 2015). When compared with groups treated with cultivated *C. vulgaris* and commercial *C. vulgaris*, group treated with cultivated *C. vulgaris* had a similar preference while the group

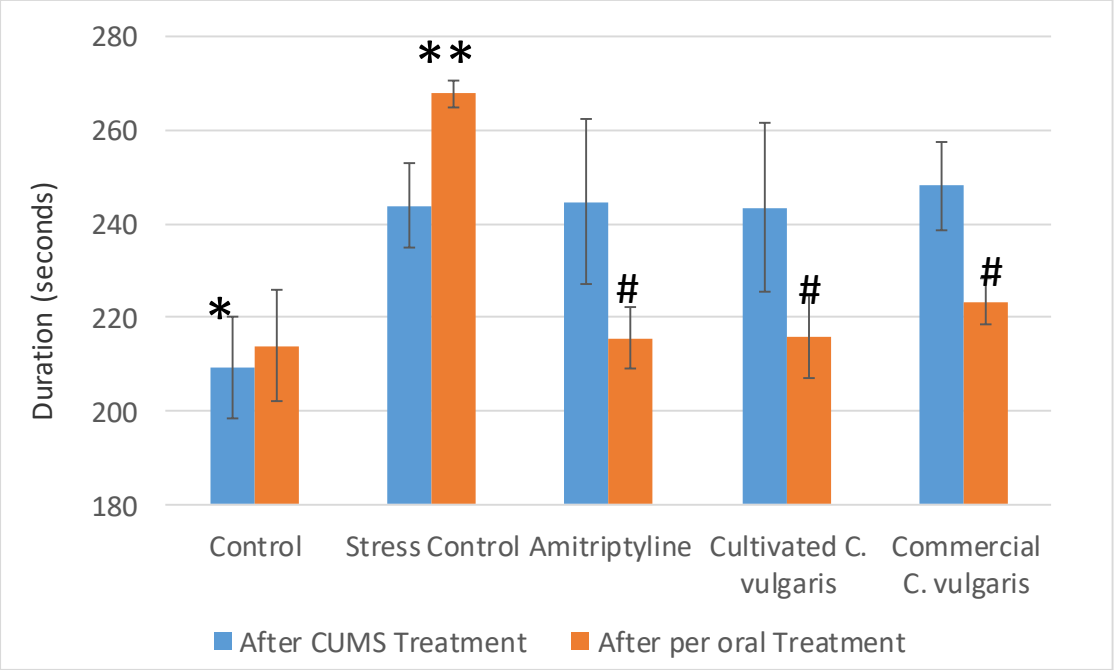


Figure 4. Immobile duration during FST on female Wistar rats after CUMS treatment and *per oral* treatment. * $p < 0.01$ compared with stress control and *per oral* treated groups; ** $p < 0.01$ compared with control and *per oral* treated groups # $p < 0.01$ compared with the same group respectively after CUMS treatment; one-way ANOVA followed by Duncan *post-hoc* test.

treated with commercial *C. vulgaris* had a higher preference value. This indicates that both type *C. vulgaris* can reduce anhedonia behaviour in animal models.

Forced Swim Test

Forced Swim Test (FST) is a common behavioural test on rodents, conducted with the aim of knowing whether a substance or a treatment has the antidepressant effect.

Based on the results on Figure 4, it was discovered that the control group had significantly (F=5.415; df=4.18; $p<0.01$) shorter duration of immobility compared with other groups. This duration has not differed much on either stress period or treatment period, which showed the consistency of animal conditions in this group throughout the study.

The negative control group had an increased immobile duration from the stress period to the treatment period. This was different from the group with *per oral* treatment, all of which showed a decline (F=28.576; df=4.18; $p<0.01$). Immobile behaviour on FST indicates despair in rodents when it realized its effort to escape from the tank is in vain, and this behaviour constitutes synonymous behaviour observed in depressed humans (Belovicova *et al.,* 2017). Amitriptyline treatment as an antidepressant caused a decrease in the duration of immobility. The same decline was observed in the group with microalgae treatment. These results indicate both type *C. vulgaris* has the potential to reduce the duration of immobility behaviour on FST.

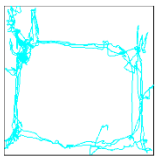
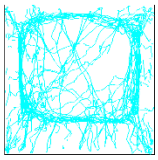
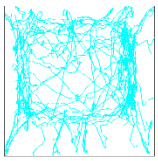
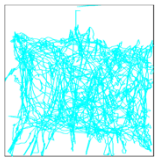
Open Field Test

Open Field Test (OFT) has been used extensively to measure anxiety behaviour in rodents. The principle of this test involves experimental animals in an unknown environment and observing the response of animals to this condition. Rodents generally choose to be on the edge and sides of the arena, avoiding the centre, unless given a substance with an anxiolytic or similar effect. This test was carried out in this research, in addition to give a more complete picture of the psychological condition of experimental animals, also because the symptoms of anxiety and depression are often times similar to each other (Tiller, 2013). The roaming of the animals was projected into a pattern and each pattern was quantified following the scale provided in Table 1.

From Figure 5 it was stated that control group has a lower roaming value than other groups, significantly in stress conditions (F=24.657; df=4.18; $p<0.01$) but not significant for animals with amitriptyline treatment at the end of study (F=15.933; df=4.18; $p<0.01$).

Chronic stress generally decreases the level of animal activity in the new environment, and this symptom can be eliminated by using antidepressants (Wang *et al.*, 2014). Group treated with microalgae produce more complex roaming patterns compared to the stress control group, but not significant

Table 1. Quantification of the trajectory pattern of rats in the OFT

Scale	Pattern	Interpretation
5		The pattern that is formed is thick on the edge and empty in the middle
4		The pattern that is formed is thick on the edge and slightly in the middle
3		The pattern formed is relatively balanced between the middle and the edges
2		The pattern that formed has many lines in the middle, on the edge is not too thick
1		Thick pattern throughout the image

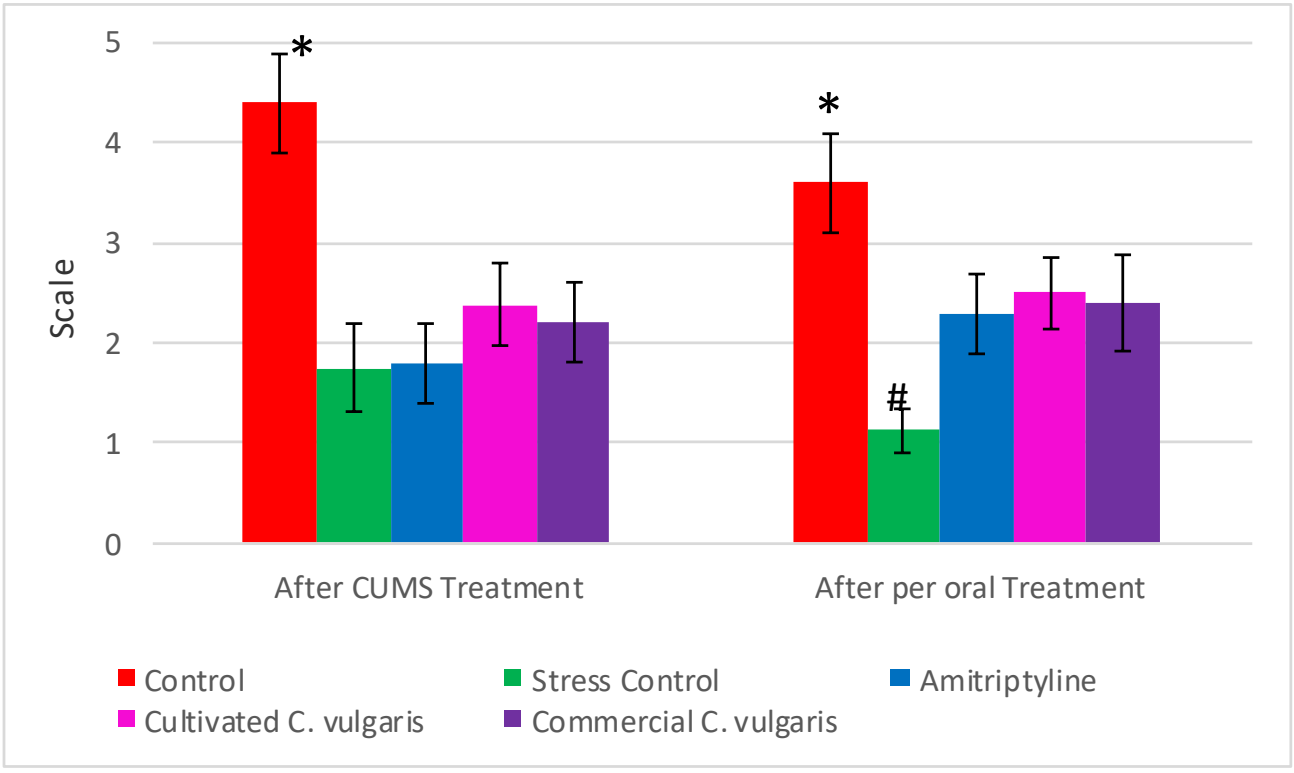


Figure 5. Comparison of the roaming behaviour of female Wistar rats in OFT after CUMS treatment and per oral treatment. *p< 0.01 compared with stress control and *per oral* treated groups; #p<0.01 compared with control and *per oral* treated groups.

Table 2. Adrenal gland of female Wistar rats after 56 days of study.

Parameters		Control	Stress Control	Amitriptyline	Cultivated *C. vulgaris*	Commercial *C. vulgaris*
Adrenal Weight (grams)		0.045±0.012^{x}	0.067±0.005^{z}	0.052±0.004^{xy}	0.055±0.01^{xyz}	0.063±0.008^{yz}
Adrenosomatic Index (%)		0.020±0.006^{x}	0.035±0.004^{y}	0.028±0.003^{y}	0.029±0.002^{y}	0.032±0.004^{y}
Cortex Composition (%)	Zona Glomerulosa	11.76±0.91^{yz}	12.40±1.71^{z}	10.53±1.33^{xy}	10.34±0.55^{xy}	9.66±0.75^{x}
	Zona Fasciculata	70.66±2.3^{x}	75.80±4.18^{y}	77.27±2.59^{y}	78.49±2.25^{y}	76.87±2.37^{y}
	Zona Reticularis	17.64±2.02^{y}	11,66±1,07^{x}	11.30±1.01^{x}	10.45±1.09^{x}	12.57±1.18^{x}
Cortex Medulla Ratio		48.23±5.28^{x}	51.73±7.18^{x}	47.45±6.1^{x}	51.73±4.15^{x}	52.76±4.83^{x}

x, y, z: different notation indicates significant difference at $p<0.05$

compared to the amitriptyline treatment group. This could mean that microalgae treatment both cultivated and commercial has the potential to increase motor activity in the OFT.

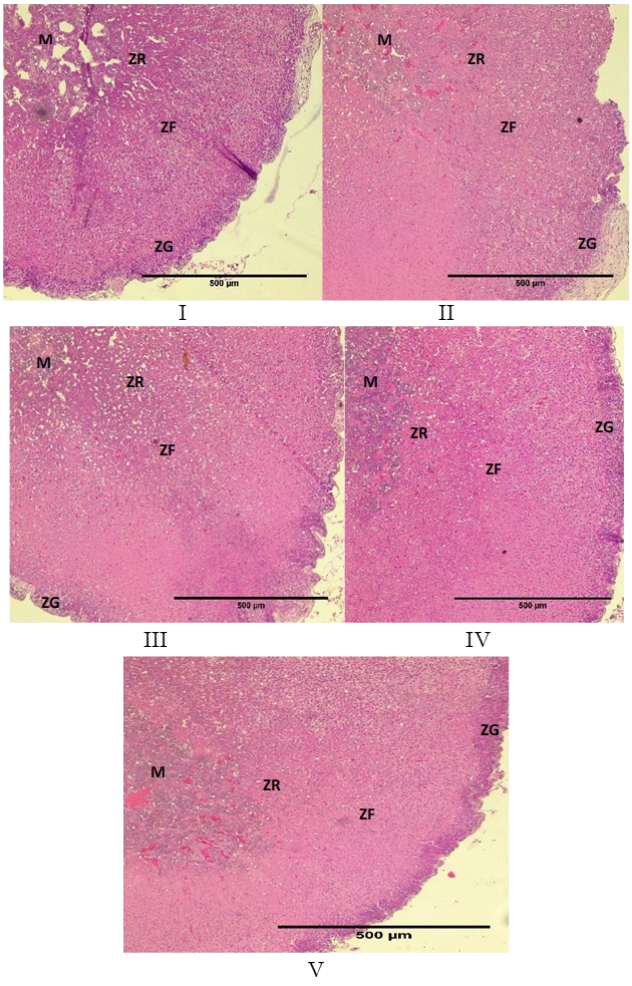


Figure 6. Histological observation of adrenal gland (100 magnification, HE stain). (I: control; II: stress control; III: amitriptyline; IV: cultivated *C. vulgaris*; V: commercial *C. vulgaris*. M: medulla; ZR: zona reticularis; ZF: zona fasciculata; ZG: zona glomerulosa).

Adrenal Glands

Stress experienced by animals can increase its adrenal gland weight due to the role of this gland in producing the hormone ACTH. This increase in weight is caused by hyperplasia and adrenal hypertrophy, reflecting the overwork of this gland (Ulrich-Lai *et al.*, 2006).

Table 2 stated that the adrenosomatic index of animals in the control group was significantly lower than the other groups (F=3.987; df=4.18; $p<0.05$). The negative control group has the largest index, however, not significant with *per oral* treated group. No significant results between stress control groups and the amitriptyline treatment group were possible because the drug had not taken effect structurally. Zoladz *et al.* (2013) found a decreased adrenal weight compared to the stressed rat after 1 month period of amitriptyline administration. The same fact could possibly be the reason why both type *C. vulgaris* failed to give a significant effect.

Based on histological observations, it was found that the percentage of zona fasciculate in the group with CUMS treatment was significantly greater (F=4.473; df=4.18; $p<0.05$) than the control group, but zona reticularis (F=18.05; df=4.18; $p<0.05$) was significantly smaller (Table 2, Figure 6). Zona fasciculata is where the hormone cortisol formed, which is stimulated by the ACTH hormone from the anterior pituitary. Cortisol plays a role in stress responses such as increasing blood pressure and energy metabolism. Chronic stress causes an increase in cortisol production so that the fasciculata zone enlarges (Ulrich-Lai *et al.*, 2006). Zona reticularis plays a role in reproduction system by producing sex hormone. Stressful events may lead to the inhibition of bodily systems which do not directly support survivability, causing energy supply to these systems to reduce until the stress has passed away (Yau & Potenza, 2014). The ratio between the cortex and medulla between groups is not significantly different. This is presumably due to enlargement of the cortex followed by enlargement of the adrenal medulla. The adrenal medulla is a place for the conversion of tyrosine amino acids into catecholamines such as epinephrine and norepinephrine. These catecholamines function in the fight or flight response and cause reactions in the body such as increased heart rate, blood pressure, narrowing of blood vessels in the skin and the gastrointestinal system and increased metabolism.

Prolonged stress will cause over-activation of the system and can cause an increase in mass in the adrenals (Ulrich-Lai *et al.*, 2006).

Brain cells are prone to damage from lipid peroxidation because most of the neuronal cell membranes are composed of unsaturated fatty acids which are the main substrates of ROS. Endogenous antioxidant defence can naturally reduce the production of ROS but chronic stress from the CUMS method is able to damage the endogenous antioxidant defences. This caused an increase in lipid peroxidation and has been studied to be one of the contributing factors to depression (Che *et al.,* 2015). *C. vulgaris* has been studied to have high antioxidant content (Saranya *et al.,* 2014) and is thought to be the cause of the antidepressant effect observed in this study. Furthermore, *C. vulgaris* is rich in tryptophan and tyrosine (Santhanam, 2015), the precursor to the neurotransmitter serotonin and noradrenaline (Hemat, 2004). These neurotransmitter's low concentration in the brain has been linked to depression as well (Lopez-Munoz & Alamo, 2011; Moret & Briley, 2011)

CONCLUSIONS

The conclusion of this study is the administration of *C. vulgaris* 360 mg/kgBW *p.o.* in female Wistar rats for 14 days after the CUMS period for 42 days was able to reduce depression-like behaviours, but was not able to revert adrenal glands size back to normal. *C. vulgaris* source did not make a notable difference to the antidepressant effects in this study. The potential of *C. vulgaris* as an antidepressant was thought to originate from its high antioxidant content which able to reduce oxidative stress in brain cells.

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Research Article

Distribution Record of *Leptophryne borbonica* (Tschudi, 1838) (Anura: Bufonidae) from Malang, East Java: Description, Microhabitat, and Possible Threats

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ABSTRACT

Bromo Tengger Semeru National Park (TNBTS) which partly located in Malang, East Java, Indonesia holds various kinds of niches that can support the existence of undiscovered amphibian species. We examine a new distribution of the *Leptophryne borbonica*, Hourglass-toad from an area located on the slopes of the Southwest of Tengger Mountain as well as its ecological implications related to the possibility of habitat threats. The exploration was carried out on January 1st and March 1st, 2019, with the description of morphology data and collecting abiotic parameters such as temperature, humidity, altitude, and habitat preference. The distribution of the species was marked. Any important notable records of the habitat threats are documented. The results showed that the amphibians found were *Leptophryne borbonica*, and set as a new record on Malang, East Java. We suggested that the isolated distribution is very susceptible to ecological disturbances, future ecotourism development, and habitat destruction that prone to local extinction. Further research and conservation efforts need to be carried out for the sustainability of this species in the observation site.

INTRODUCTION

Amphibians diversity data in East Java is still recorded on several localities (van Kampen, 1923) with a lack of further survey afterward. Previous data shows only 39 species of amphibians (mostly dominated by amphibian species of Anura, and 3 species of Gymnophiona) (Iskandar & Colijn, 2000) in the island of Java, with vast majority data originating from West Java region, especially on the Mount Halimun-Salak National Park and Gunung Gede Pangrango National Park (Iskandar, 1998; Mumpuni, 2001; Kusrini, 2007), and several regions in the Central Java province especially on the Mount Slamet region (Riyanto, 2010). Several attempts to collect amphibian diversity studies have been carried out in East Java, especially in Batu, Malang, Kediri, and other regions (Septiadi et al., 2018b; Hanifa et al., 2016; Indrawati et al., 2018; Hidayah et al., 2018), although more exploration needs to be carried out. The amphibians diversity data in East Java are very important in assessing its distribution, conservation status and strategies to prevent the threat of declining population.

Malang as the largest regency in East Java has an administrative area of 3,534.86 km², was surrounded by mountain ranges and a lot of watersheds (Pemkab Malang, 2019). This area has high potency of biodiversity, but still minimal in term of exploration and data of herpetofauna. One area that has high biodiversity potential is Bromo Tengger Semeru National Park (TNBTS) and its surroundings. This region keeps various niches such as high ground, water source, clear water flow, low to high canopy & dense vegetation, and also plenty of habitat such as arboreal, terrestrial, fossorial, and aquatic type that might support the existence of amphibian species (Septiadi et al, 2018b). This area has a tourism attraction (ecotourism) which

indirectly impacting on the sustainability of ecosystems, niche and the diversity of species in it.

Leptophryne borbonica (Tschudi, 1838) or Hourglass-toad is a relatively small body toad that has specific hourglass pattern on its back. This species was distributed only in Borneo, Sumatra, Peninsular Malaysia, and also Java Island with type locality from West Java (Iskandar, 1998), and also was recorded in Tengger Mountain, without exact location (Ardiansyah et al., 2014; Iskandar & Colijn, 2000). Other species found in this genus is *Leptophryne cruentata* (Tschudi, 1838) which is distributed only in West Java and Central Java (Iskandar and Colijn, 2000; Mumpuni, 2014), and newly recorded species namely *Leptophryne javanica* Hamidy, Munir, Mumpuni, Rahmania & Kholik, 2018 which is endemic, protected, and only distributed on the conservation area in the slopes of Mount Slamet and Mount Ciremai in West Java (Hamidy et al., 2018). Distribution mapping, the collection of habitat preference data, the population size of species, and the description related to the possibility of cryptic species in this genus (Hamidy et al., 2018) are very important due to the limited information about this species in East Java. Thus, we report a new distribution with a description of the Hourglass-toad from East Java region as well as its ecological implications related to the possibility of habitat threats to the sustainability of its population.

MATERIALS AND METHODS

Study Locations

The specific location (encrypted) is hidden on the Southwest of one of the slopes of the Bromo Tengger Semeru National Park (TNBTS) included in Malang, East Java, Indonesia (Figure 6). The location points are buffer zones that are used as human activities, agroforestry, and nature tourism sites. The small water stream as the habitat of the species *L. borbonica*, disembogues into a large river bank which is a series of several waterfalls from upstream to downstream and encountered 24 adult and 5 tadpole species of *L. borbonica*. This study carried out on an expedition of "Malang Herpetofauna Biodiversity Project", conducted on January 1st and March 1st, 2019.

Specimens Desctiption Data

Morphological characters were observed as validation of the species description of *L. borbonica*. Eight adult specimens (7 adult males, 1 adult female) and 5 tadpoles were deposited on the Ecology Laboratory, Biology Department, State Islamic University of Maulana Malik Ibrahim Malang. Important characters are observed such as patterns and variations on the dorsum side, ventrum side, lateral side of adult specimens which then compared to literature, and also webbing diagrams and formulas (Figure 1) following Glaw and Vences (1994), and tadpole stages determination following Gosner (1960) and McDiarmid & Altig (1999).

Distribution and Habitat Data

The survey was conducted using Visual Encounter Survey (VES) method combined with Time Search Sampling method (Hill et al., 2005) starting at 6-12 pm, with only 4-5 person involved. The species distribution mapped out by traversing the water stream as the specific niche of *L. borbonica*, then divided into 3 plots based on stream position (upstream, middlestream and downstream) which disembogues at the bank of a larger river with a length of small watershed approximately ± 45 m (± 15 m each plot). Observed specimen was marked and the elevation was recorded using Garmin GPSMAP 64s, then the distribution was projected (Figure 2) using QGIS Desktop 2.18.12. The habitat preferences of each individual found in each plot were documented, air temperature and humidity (Table 1) were also recorded using a Thermo hygrometer as a specific habitat reference, and the possibility of predatory species from *L. borbonica* was also noted along with the observation plot.

RESULTS AND DISCUSSION

Specimen Description

Description based on the observation of adult male of *Leptophryne borbonica* as follows: A small size toad (♂ SVL = ± 23.5 mm; ♀ SVL = 26.10 mm), habitus slender, with long forelimbs and a short hindlimbs; bony crest absent, snout projects slightly over the mouth in profile, the tips of finger are rounded (not dilated), firmisternal pectoral girdle present after dissection, paratoid glands indistinct, dorsum with black hour-glass marking, tympanum distinct, a median subgular vocal sac and vocal slit are present on males; skin above wrinkled and scattered on all parts including dorsal surface on the forelimbs and hindlimbs, supratympanic fold absent; Ventrum weakly granulated especially on chest, nuptial pads present on male first finger. Coloration including brown dorsum mottled with black spots on head, back, and limbs; groin and ventral surface of forelimbs and hindlimbs reddish, and webbing are both dorsally and ventrally reddish; belly with suffusion of black and white tend to brownish, chest and throat blackish, limbs with distinct black bars dorsally, upper lip with black bars; pupil horizontal, iris golden with netted black pattern (Figure 1 & 2). The webbing includes hindlimbs with web formulas

Figure 1. Life photograph of adult male of *Leptophryne borbonica* from the slopes of the Southwest of Tengger Mountain (Photographed by Luhur Septiadi).

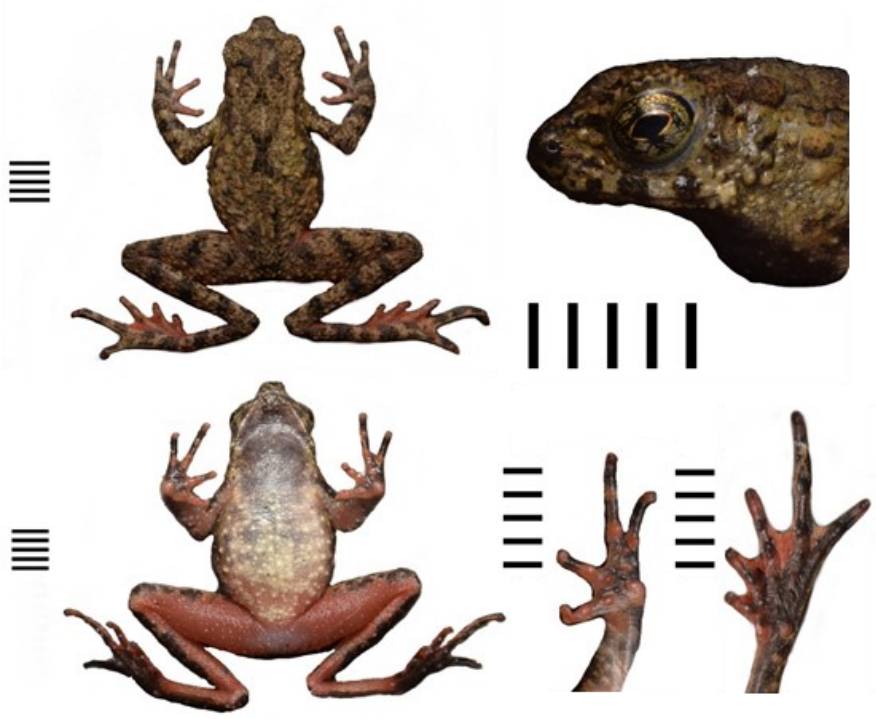

Figure 2. Dorsum and ventrum views (left side), and lateral view of the head, ventral surface of the left hindlimb and left forelimb (right side) of male *Leptophryne borbonica* from the slopes of the Southwest of Tengger Mountain. Scale bars = 5 mm (Photographed by Muhamad Prayogi Erfanda).

as follows: I 0-1 II 0-2 III 1-3 IV 2¾-1 V (Figure 4), forelimbs webbings are restricted to the base.

The morphological characters observed are mostly suited to the description of *L. borbonica* (Inger & Stuebing, 2005; Hamidy et al., 2018) with similar web formulas from Hamidy et. al (2018) (I ½-1 II 1-2 III 1½-3 IV 3-1 V; our direct examination: I 0-1 II 0-2 III 1-3 IV 2¾-1 V), with no black triangular marking behind the eyes, unlike stated by Iskandar with black triangular on some specimens from another localities (Iskandar, 1998)

Microhabitat Preference and Distribution

A total of 24 specimens of *L. borbonica* were recorded, often found descend on moist rock substrates (97-99% humidity) on the banks of the slow water stream, damp cliff edges with vegetation of bryophytes mosses (Figure 5), and there none to be found perched on tall leaves or trees (some species perched on low leaves, height approximately 0–1.9 meters from the ground). According to Iskandar (1998), it was found abundant around wet areas or in clear, slowing-moving waters. According

Figure 3. Photos of *Leptophryne borbonica* preserved specimens of tadpole hatchling. Dorsum views (left side), lateral views (upper right side), ventral views (lower right side) of tadpole at hatchling phase of stage 24th from the slopes of the Southwest of Tengger Mountain. Scale bars = 1 mm (Photographed by Muhamad Prayogi Erfanda).

to Inger & Stuebing (2005), this small toad often found in the leaf litters of seepage areas in forests.

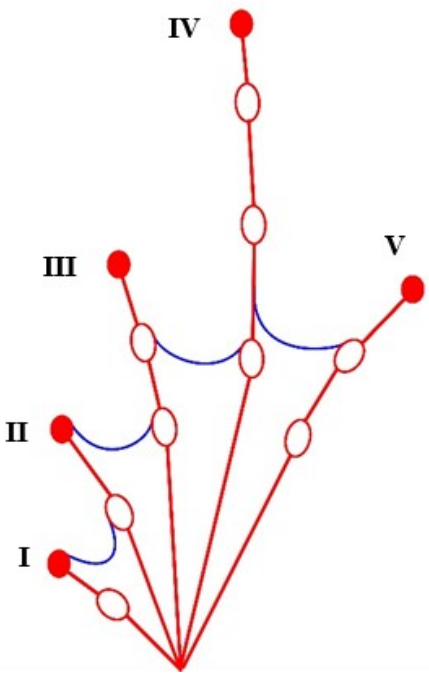

Figure 4. Webbing formula of left hindlimbs of male *Leptophryne borbonica*, number I - V indicated the inner to outer finger, webbing formulas as follows: I 0–1 II 0–2 III 1–3 IV 2¾-1 V (illustrated by Muhamad Prayogi Erfanda).

Some tadpoles of *L. borbonica* were managed to observe on calm flowing ponds. The tadpoles were obtained at the stage 20th (with no pigmentation, tends to be white translucent, a presence of gill circulation, elongation of tail). Some specimens were taken to the laboratory to clarification and identification purpose. The tadpoles were photographed at the stage 24th-25th (with development of operculum, oral disc, and pigmentation) (Figure 3) with only one male specimen managed to become adult male. We collected as much as 7, 9, 8 adult individuals respectively encountered in the upstream, middlestream, and downstream, and no species to be found out of the observed plot (> 45 m). The air temperature recorded in the upstream, middlestream, and downstream were ± 19^{0}C, ± 20^{0}C, ± 23^{0}C respectively, indicating an increase in air temperature towards the downstream. Humidity recorded at the upstream, middlestream, and downstream were ± 97 %, ± 98 %, ± 99 % respectively, indicating the higher humidity towards the downstream (Table 1). These physical parameters conclude that *L. borbonica* observed to be in varying range of air temperature 19-23^{0}C and varying humidity range 97–99 %. The species was mostly found in the middlestream of the water flow, and it is difficult to conclude the correlation only by the physical parameters. According to Ardyansah et. al (2014), the abundance of *L. borbonica* has a weak correlation with physical factors (temperature, humidity, and salinity) at the study location. However, the slightly different in the number of species encounter, indicated that there were other factors that may responsible for this issue. The species abundance indicated to be influenced by the current flow, where this species prefers the calmly

Figure 5. The habitat of *Leptophryne borbonica* from the slopes of the Southwest of Tengger Mountain: (A) upstream habitat, (B) a closer look at *L. borbonica* on upstream habitat, (C) a species of *L. borbonica* at downstream habitat; red circle indicated the species-specific location (Photographed by Luhur Septiadi).

flows water for mating purpose, which are mostly found in the middlestream.

Table 1. The physical parameters recorded from each observed plot of *L. borbonica* niche

Water stream	Air temperature	Humidity	Individual
Upstream	± 19 ^{0}C	± 97 %	7
Middlestream	± 20 ^{0}C	± 98 %	9
Downstream	± 23 ^{0}C	± 99 %	8
Range	19 – 23 ^{0}C	97 - 99 %	± 8

Distribution mapping shows that the presence of *L. borbonica* is very isolated (± 45 m) and currently recorded on a specific location (Malang) especially in East Java. The distribution of *L. borbonica* can only be found at an elevation of ± 870-850 m.a.s.l. based on the observed location. According to Hamidy et. al (2018), *L. borbonica* occurs in both lowlands and highlands up to 1400 m.a.s.l, while according to Inger & Stuebing (2005), this toad is most often found below 400 m.a.s.l. The presence of *L. borbonica* is limited by a very steep niche in the upstream area (Figure 5a) and ends with a stream of water that has seeped into the rocks on the downstream area, disembogues into a large river of waterfall stream (Figure 6).

Habitat Threats

The *L. borbonica* habitat is threatened by many natural factors, such as predators. Other Anurans species were found, such as *Limnonectes microdiscus*, *Odorrana hossi,* and *Megophrys montana* which might act as natural predators for *L. borbonica*. Bigger size frogs tend to prey on smaller vertebrate animals (including frogs) in their dietary performing anurophagy. But, the circumstance under which frogs eat another frog are likely to be complex (Measey et al., 2015), further

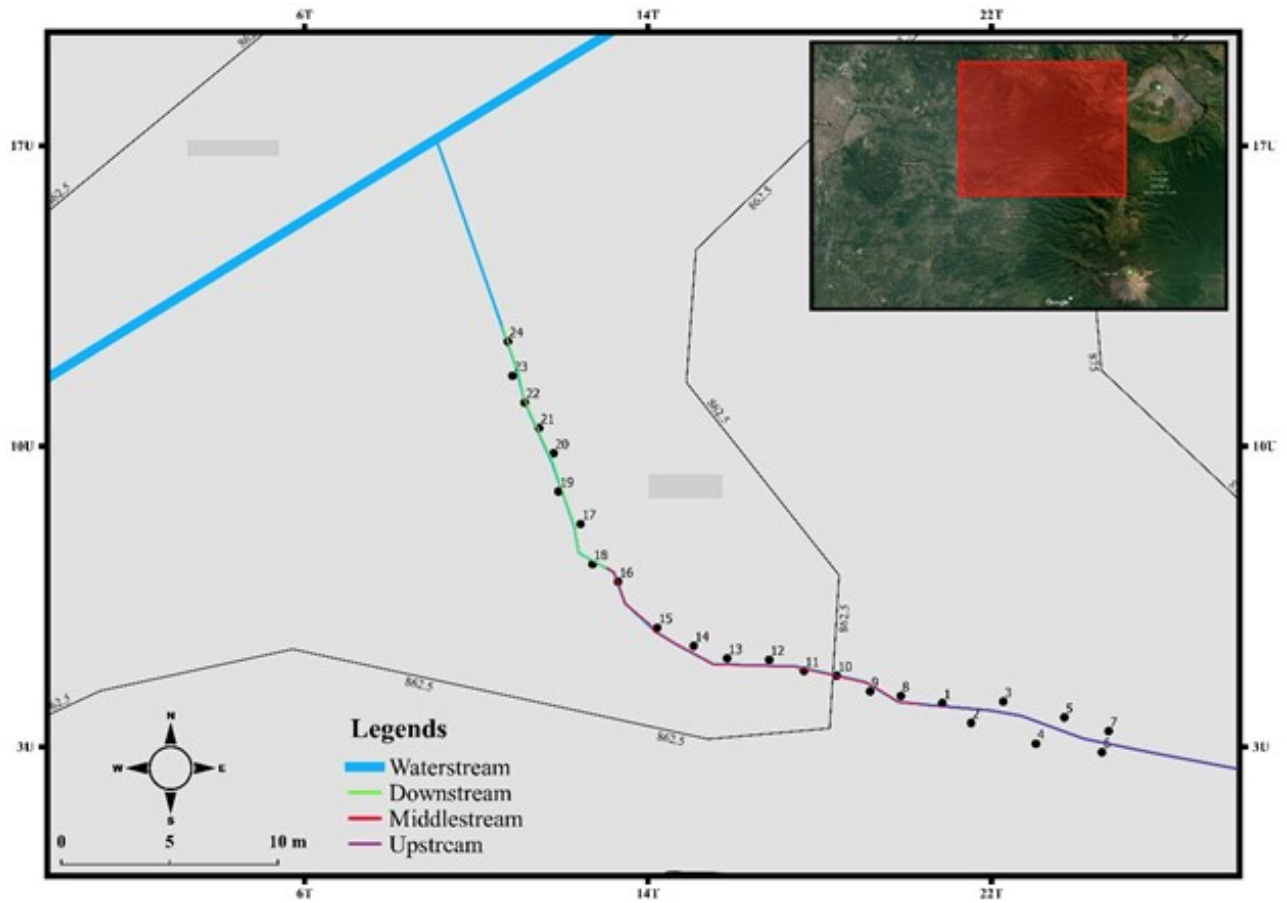

Figure 6. The records location for *L. borbonica* from the slopes of the Southwest of Tengger Mountain (specific location is encrypted), the number indicated the sequence of specimens encounters, the elevation is indicated by contours, plots is are distinguished by color (projected by QGIS Desktop 2.18.12).

research on dietary (via digestive organ examination) and the interspecific relationship of each species that live in the same niche are yet to be studied. Another habitat threats, include narrow distribution on their specific habitats niche (found only at ± 45 m) and might be susceptible by the landscape change. Massive landscape changes such as volcanic activities are likely resulting on regional extinctions of this species in East Java. According to IUCN (2014), deforestation is a major threat for this species. Forest clearing for agricultural activities may only have a small impact on the population because they leave the subpopulations to re-invade secondary forest nearly. The species are also threatened by future volcanoes activity of Bromo Tengger Semeru mountain, although this species is widely distributed in Java region (Iskandar, 1998). The nature tourism development will also contribute to habitat loss and disease transmission (Septiadi et al., 2018a), exposing to the infecting chytrid fungus as reported by Kusrini et. al (2008), and a reduction in population size due to the development ecotourism that poorly managed and monitored given its location adjacent to the location of waterfalls. More research and conservation efforts need to be carried out to maintain the existence of the population of *L. borbonica* in the East Java region.

CONCLUSIONS

New amphibian distribution recorded on the slopes of TNBTS that partly located in Malang, East Java was *Leptophryne borbonica* (Hourglass-toad) and set as a new record in the East Java region. Its isolated distribution is very susceptible to ecological disturbances, future ecotourism development, infectious disease, population loss, and prone to local extinction. Research data and conservation efforts need to be carried out for the sustainability of the species. Further molecular, morphological and vocalization analysis is also needed to confirm this species of amphibians.

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