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**Title:**

Genetic Diversity of *Phaseolus lunatus* L. in East Java based on RAPD Marker

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## GENETIC DIVERSITY OF *Phaseolus lunatus* L. IN EAST JAVA BASED ON RAPD MARKER

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### ABSTRACT

*Phaseolus lunatus* L. is one of legume plants that can be found at some parts of Indonesia and is potential for alternative food rich of protein. This current research aimed at analyzing genetic accessions of *P. lunatus* distributed at some areas in East Java, Indonesia based on RAPD marker. A total of 15 accessions originated from Tulungagung, Kediri, Malang, Probolinggo, and Madura were analyzed. In addition, there were 10 primers used, i.e. OPA6, OPA8, OPA10, OPA20, OPC19, OPD8, OPA10, OPA20, OPC19, OPD8, OPE15, and OPE16 generating 68 lines of DNA band typified with different sizes. Further, there were 9 primers in the possession of 100% level of polymorphism in which only Primary OPC19 yielded 96% level of polymorphism. By means of Neighbor-Joining method, a phylogenetic tree was yielded by similarity coefficient of 64-100%. On the genetic similarity coefficient of 0.6 in *P. lunatus* accessions, there were two groups, the first and second major clusters, Cluster A and B. The former contained the accessions 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Keywords:** Genetic diversity, *Phaseolus lunatus*, RAPD.

## INTRODUCTION

*Phaseolus lunatus* L. is categorized as a legume plant with its great potential to become nutritious food. This kind of plant is also known as *koro* beans or lima beans and is identical with its vital nutritious contents, from protein (Chel-Guerreoro *et al.*, 2002; Betancur-Ancona *et al.*, 2003; Bello-p *et al.*, 2007; Tejasari, 2016) to fiber (Betancur-Ancona *et al.*, 2003; Segura-Campos & Betancur-Ancona. 2010; Betancur-Ancona *et al.*, 2004; Ancona *et al.*, 2011; Novelo-cen & Betancur-Ancona, 2005). In addition, this plant also contains extra nutrition stored at its stipule or stalk (Heil, 2004; Kost & Heil. 2005). In addition to its richness of nutritional contents, *P. lunatus* L. is also reported to contain a number of anti-oxidant compounds (Betancur-ancona *et al.*, 2004; Da *et al.*, 2009) and other substances that are effective to optimize human's health (Bello-p *et al.*, 2007; Da *et al.*, 2009; Wong & Ng, 2005; Al *et al.*, 2007). For that reason, not only is *P. lunatus* L. known as protein-sourced food in many countries (Almeida & Pedrosa-Harand, 2011; Giami, 2001), it is also reported to be effective in preventing various illnesses (Bello-p *et al.*, 2007; Da *et al.*, 2009; Wong & Ng, 2005; Al *et al.*, 2007).

In addition to its benefits, *P. lunatus* L. can grow in some patterns of conditions (Lim, 2012). Therefore, it is common if *P. lunatus* can be found in many parts of countries as it has been domesticated there (Chacón-Sánchez & Martínez-Castillo, 2017; Félix *et al.*, 2014; Camacho-Pérez *et al.*, 2018). In Indonesia, to be specific, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti & Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions to exist in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as alternatively functional food source (Nafi *et al.*, 2015; Nafi *et al.*, 2006; Kalaminasih, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2014; Diniyah *et al.*, 2013). Now that Indonesia is dealing with serious issue of protein shortage in some particular areas (Ickowitz *et al.*, 2016; Fatmah, 2005; Diana *et al.*, 2017; Madanijah *et al.*, 2016), maximizing the consumption of *P. lunatus* will be of effectiveness for the solution.

With respect to the attempt of elevating the use of *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content is in need of actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential

foundation to run all the program of food source enhancement (Bhanu, 2017; Mohammadi & Prasanna, 2003). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a scheme of betterment for the species since the beginning of the period of systematic plant breeding (Bhanu, 2017). Furthermore, such kinds of information can be used to reach a phase of sustainable crop production (Fu, 2015). What is more, a research underpinning genetic diversity in particular plant also leads to an attempt of conservation (Carvalho *et al.*, 2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using such numerous techniques as morphological identification, biochemical characterization, and analysis of DNA marker (Govindaraj *et al.*, 2015).

With reference to the existence of those techniques, the selection of molecular marker is considered more appropriate and effective, not only to avoid any bias due to environmental influence but also to provide eclectic information related to genetic diversity in more acceptable way (Fu, 2015). There are some molecular markers included and considered particularly promising in helping analyse genetic diversity, namely RAPD, RFLP, and SCAR.

Amongst those markers, RAPD is defined as the most popular marker in many researches (Ben-Ari & Lavi, 2012). RAPD constitutes PCR-based technique which involves primary set with relatively short size and is able to randomly amplify many DNA segments (Kumari & Thakur, 2014). This technique is equipped with notable excellence in comparison with other techniques, which is that the technique occupies a universal primary set without undergoing DNA sequencing phase in its real implementation (Ali *et al.*, 2004). In addition, RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar & Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari & Thakur, 2014), and is applicable for various laboratory situations (Kumar & Gurusubramanian, 2011). RAPD, therefore, was used in this current research in order to study genetic variation of various legumes, from *P. vulgaris* to *P. lunatus*.

With respect to the aforementioned rationale, analyses of genetic diversity of *P. lunatus* in Indonesia are still of rarity. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in Africa (Fofana *et al.*, 2001), Europe (Sparvoli *et al.*, 2001), North America (Fofana *et al.*, 2001; Serrano *et al.*, 2010), Central America (Bi *et al.*, 2005) Latin America (Fofana *et al.*, 2001; Serrano *et al.*, 2010) and Caribbean countries (Fofana *et al.*, 2001). On one hand, researches in Indonesia are still limited to study on the potential of *P. lunatus* as alternative food source (Herry *et al.*, 2014; Diniyah *et al.*, 2013) alongside its essential substances (Tejasari, 2016; Nafi *et al.*, 2006; Diniyah *et al.*, 2015; Praseptiangga *et al.*, 2018; Sukatiningsih *et al.*, 2013). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti & Fauzi, 2019; Waluyo *et al.*, 2016). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on RAPD marker.

## MATERIAL AND METHODS

### Plant material

*P. lunatus* used in this research was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. On the basis of the result of identification in previous research (Purwanti & Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Each of the accessions, further, was planted in a polybag in which one polybag distanced one meter long to the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agent.

**Table 1.** List of accessions to analyze

### DNA Isolation

DNA isolation was administered based on CTAB method of Doyle & Doyle (1984), which was modified by Maftuchah & Zainuddin (2010). The used tissue was stemmed from leaf organ of 3-month-old plant. The followings were the procedures of DNA isolation: **1)** leaves were cut out into 2-3 pieces with the length of 1-2 cm; **2)** 0.3-gram leaves and liquid nitrogen were crushed using mortar and pestle; **3)** 0.002-gram Natrium bisulfite was weighed for each of 12 samples; **4)** 0.02-gram Na bisulfite was dissolved into 9,600- $\mu$ l buffer (labelled Buffer I); **5)** label was given to each 1.5-ml tube based on the name of the sample; **6)** Buffer I mixed with 800- $\mu$ l Natrium bisulfite was poured into each tube; **7)** the crushed sample was added to each tube; **8)** the tube was shaken until the crushed leaves were submersed into the buffer; **9)** the sample was incubated under the ideal temperature with the span of  $\pm$  5 minutes; **10)** the sample was centrifuged under the speed of 12.000 rpm, the temperature of 4°C with the span of 10 minutes; **11)** supernatant was eliminated; **12)** 500- $\mu$ l buffer II was added to the natant placed in the tube; **13)** the tube was vortexed; **14)** the sample was incubated under the temperature of 65°C with the span of 30 minutes while flipping over the tube for every 10 minutes during the incubation; **15)** chloroform was added: alcohol isoamyl (24:1) with the volume of  $\pm$ 500 $\mu$ l; **16)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **17)** the supernatant was taken and put into new tube; **18)** absolute ethanol with the volume of  $\pm$  800 $\mu$ l was added along with natrium acetate with the volume of 80 $\mu$ l while doing homogenization through flipping over the tube all around; **19)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **20)** the supernatant was eliminated; **21)** 70%-concentrated ethanol with the volume of  $\pm$  800  $\mu$ l was added; **22)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **23)** the DNA pellet was dried out; **24)** Tris-EDTA

(TE) with the volume of 60 µl was added into tube; **25)** the results of isolated DNA were stored under the temperature of -20°C.

### **PCR-RAPD**

In the reaction of PCR, the isolated DNAs of *P. lunatus* L. were used as the mold. There were 10 kinds of primers used such as OPA6, OPA8, OPA10, OPA20, OPC19, OPD8, OPD12, OPE8, OPE15, and OPE16. The total volume of PCR reaction used signified 22.5 µl, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and molded DNA. The condition for PCR reaction was designed under pre-denaturation temperature of 94°C (in 5 minutes), denaturation temperature of 94°C (in 1 minute), primary attachment temperature of 36°C (in 1 minute), extension temperature of 72°C (in 2 minutes), post-extension temperature of 72°C (in 5 minutes), and post PCR reaction temperature of 4°C (in 2 minutes). For multiplication, the cycle of PCR reaction was repeated 36 times.

### **Electrophoresis**

There were three stages of procedure to confirm the result of isolation process as well as PCR reaction after implementation. The first stage was the creation of agarose gel with the concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labeled electrophoresis with the electrophoresis buffer of TBE (1x), loading dye (6x) under the condition of 60 V, 400 mA within 45 minutes. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

### **Analysis on the DNA bands yielded from RAPD**

Data analysis was performed by observing the pattern of visible bands from electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data indicating the existence and inexistence of bands typifying certain sizes. Afterwards, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and to create a phylogenetic tree by the assistance of Popgen software version 3.1.

## **RESULTS AND DISCUSSION**

In this current research, as many as 10 primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, the bands, as a result of PCR–RAPD amplification at those genotypes (originated from East Java), were assessed based on the binary data, with the description of: 1 for amplified band and 0 for unamplified. The following Table 2 shows the record of the number of locus found.



**Table 2.** The record of the number of locus found at 15 genotypes of *P. lunatus* L using 10 random primary

10 primers used to amplify the DNA at 15 genotypes of *P. lunatus* L. could reproduce DNA fragments with diverse sizes (polymorphic) despite its capability of yielding 68 DNA band lines (Table 3).

**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.

Variation of genotype diversity at 15 genotypes resulted in 68 RAPD band assessed from 10 primary oligonucleotide with the means of 6.8 bands per primary. Out of the 10, 68 bands were yielded with various sizes of DNA fragments. The result of amplification showed that almost all bands indicated similar pattern of DNA band, polymorphic. The primary with the smallest number of bands was primary OPA10 and OPA20; while the one with the highest number of bands was OPD12. Moreover, there were 9 primers with 100% level of polymorphism. To be specific, OPC19 was only effective to result in 96% level of polymorphism.

Next, cluster analysis upon the 68 RAPD bands was administered. This phase yielded a phylogenetic tree by the assistance of Neighbor-Joining method, equipped with similarity coefficient that ranged from 64%-100%, or there was genetic variation with the range of 0-36% (Figure 1). In genetic similarity coefficient of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with genetic similarity coefficient of 0.932 (including 8, 12, 13, 14 genotypes), A2 with genetic similarity coefficient of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). With respect to that with genetic similarity coefficient of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close. In addition, a phylogenetic tree constructed on the basis of RAPD analysis is shown in Figure 1.

**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* under the study was relatively high (ranging from 96 to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Chacón-Sánchez & Martínez-Castillo, 2017; Camacho-Pérez, *et al.*, 2018), South America, and Afrika (Fofana, *et al.*, 1997). The existing high polymorphism had indicated the huge range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes one of a series of parameters to define genetic diversity on particular species (Snustad & Simmons, 2012). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of gene pool. According to previous study, the 15 accessions of *P. lunatus* included in current research were originated from 5 different gene pool, to name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti & Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The different ecological difference was closely interconnected with agroclimatic zone difference in which the origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity which was considered high had brought about an urgent implication in terms of crop improvement attempt, to include breeding procedure for quality improvement (Bhanu, 2017; Mohammadi & Prasanna, 2003; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not really effective as main food resource (Doria *et al.*, 2012; Offei *et al.*, 2003; Adeparusi, 2001). Consequently, the process of maturation in *P. lunatus* seeds was in need of proper and acceptable process, thus it could reduce the anti-nutritional substances (Adeparusi, 2001; Granito *et al.*, 2007; Sukatiningsih *et al.*, 2013). Further, by making use of data about genetic variety in breeding procedure, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factor. In addition, breeding procedure could be designed that way so as to yield the generation of *P. lunatus* with highly pest-resistant performance and shorter period of maturation phase.

Next, with reference to the phylogenetic tree that was formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior.

Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width and pod width in comparison to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti & Fauzi, 2019). This demonstration was in line with phylogenetic tree resulted in this current research in which Probolinggo accessions as well as Accession 4 were clustered altogether in Cluster B.

Despite the fact, the result of cluster analysis also indicated the existence of accessions of which pod lengths were quite different, but still grouped in the same cluster. In contrast, when the pod length was not significantly different, the result of cluster analysis classified those accessions in different cluster. This was probably due to DNA related to RAPD marker used in this current research was unrelated to the character setting. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, moreover, the use of RAPD was not only affordable, but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. In spite of it, there were some studies of genetic variation on germplasm of beans which were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye & Ojobo, 2012), Turkey (Ince & Karaca, 2011), and in *Vicia Faba*, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez & Martínez-Castillo, 2017). In short, this current research has advocated the credibility of the use of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

## CONCLUSION

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analyzed. The result of RAPD utilizing 10 primers resulted in 68 bands in which 9 primers possessed 100% level of polymorphism and one primary was equipped with 96% level of polymorphism. On the basis of cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura



Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

**Table 2.** The record of the number of locus found at 15 genotypes of *P.lunatus* L using 10 random primary

Codes	Locus (bp)												
	10 0	20 0	30 0	400	50 0	60 0	70 0	80 0	90 0	100 0	110 0	120 0	1300
2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

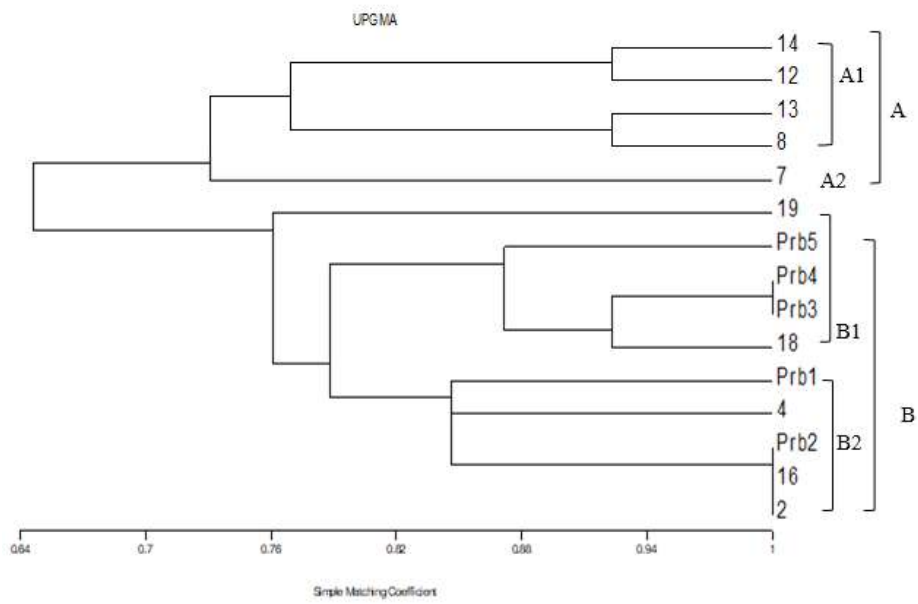
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
**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.





No.	Markers (100 bp–2000 bp)	$\Sigma$ Band	Polymorphic	% Polymorphic
1	OPA 6	8	8	100%
2	OPA 8	7	7	100%
3	OPA 10	5	5	100%
4	OPA 20	5	5	100%
5	OPC 19	6	5	96%
6	OPD 8	8	8	100%
7	OPD 12	9	9	100%
8	OPE 8	7	7	100%
9	OPE 15	7	7	100%
10	OPE 16	6	6	100%
Total		68	67	



**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

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**GENETIC DIVERSITY OF *Phaseolus lunatus* L. IN EAST JAVA BASED ON RAPD  
MARKER**

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**ABSTRACT**

*Phaseolus lunatus* L. is one of legume plants that can be found at some parts of Indonesia and is potential for alternative food rich of protein. This current research aimed at analyzing genetic accessions of *P. lunatus* distributed at some areas in East Java, Indonesia based on RAPD marker. A total of 15 accessions originated from Tulungagung, Kediri, Malang, Probolinggo, and Madura were analyzed. In addition, there were 10 primers used, i.e. OPA6, OPA8, OPA10, OPA20, OPC19, OPD8, OPA10, OPA20, OPC19, OPD8, OPE15, and OPE16 generating 68 lines of DNA band typified with different sizes. Further, there were 9 primers in the possession of 100% level of polymorphism in which only Primary OPC19 yielded 96% level of polymorphism. By means of Neighbor-Joining method, a phylogenic tree was yielded by similarity coefficient of 64-100%. On the genetic similarity coefficient of 0.6 in *P. lunatus* accessions, there were two groups, the first and second major clusters, Cluster A and B. The former contained the accessions 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Keywords:** Genetic diversity, *Phaseolus lunatus*, RAPD.

## INTRODUCTION

*Phaseolus lunatus* L. is categorized as a legume plant with its great potential to become nutritious food. This kind of plant is also known as *koro* beans or lima beans and is identical with its vital nutritious contents, from protein (Chel-Guerreoro *et al.*, 2002; Betancur-Ancona *et al.*, 2003; Bello-p *et al.*, 2007; Tejasari, 2016) to fiber (Betancur-Ancona *et al.*, 2003; Segura-Campos & Betancur-Ancona. 2010; Betancur-Ancona *et al.*, 2004; Ancona *et al.*, 2011; Novelo-cen & Betancur-Ancona, 2005). In addition, this plant also contains extra nutrition stored at its stipule or stalk (Heil, 2004; Kost & Heil. 2005). In addition to its richness of nutritional contents, *P. lunatus* L. is also reported to contain a number of anti-oxidant compounds (Betancur-ancona *et al.*, 2004; Da *et al.*, 2009) and other substances that are effective to optimize human's health (Bello-p *et al.*, 2007; Da *et al.*, 2009; Wong & Ng, 2005; Al *et al.*, 2007). For that reason, not only is *P. lunatus* L. known as protein-sourced food in many countries (Almeida & Pedrosa-Harand, 2011; Giami, 2001), it is also reported to be effective in preventing various illnesses (Bello-p *et al.*, 2007; Da *et al.*, 2009; Wong & Ng, 2005; Al *et al.*, 2007).

In addition to its benefits, *P. lunatus* L. can grow in some patterns of conditions (Lim, 2012). Therefore, it is common if *P. lunatus* can be found in many parts of countries as it has been domesticated there (Chacón-Sánchez & Martínez-Castillo, 2017; Félix *et al.*, 2014; Camacho-Pérez *et al.*, 2018). In Indonesia, to be specific, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti & Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions to exist in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as alternatively functional food source (Nafi *et al.*, 2015; Nafi *et al.*, 2006; Kalaminasih, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2014; Diniyah *et al.*, 2013). Now that Indonesia is dealing with serious issue of protein shortage in some particular areas (Ickowitz *et al.*, 2016; Fatmah, 2005; Diana *et al.*, 2017; Madanijah *et al.*, 2016), maximizing the consumption of *P. lunatus* will be of effectiveness for the solution.

With respect to the attempt of elevating the use of *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content is in need of actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential

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foundation to run all the program of food source enhancement (Bhanu, 2017; Mohammadi & Prasanna, 2003). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a scheme of betterment for the species since the beginning of the period of systematic plant breeding (Bhanu, 2017). Furthermore, such kinds of information can be used to reach a phase of sustainable crop production (Fu, 2015). What is more, a research underpinning genetic diversity in particular plant also leads to an attempt of conservation (Carvalho *et al.*, 2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using such numerous techniques as morphological identification, biochemical characterization, and analysis of DNA marker (Govindaraj *et al.*, 2015).

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With reference to the existence of those techniques, the selection of molecular marker is considered more appropriate and effective, not only to avoid any bias due to environmental influence but also to provide eclectic information related to genetic diversity in more acceptable way (Fu, 2015). There are some molecular markers included and considered particularly promising in helping analyse genetic diversity, namely RAPD, RFLP, and SCAR.

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Amongst those markers, RAPD is defined as the most popular marker in many researches (Ben-Ari & Lavi, 2012). RAPD constitutes PCR-based technique which involves primary set with relatively short size and is able to randomly amplify many DNA segments (Kumari & Thakur, 2014). This technique is equipped with notable excellence in comparison with other techniques, which is that the technique occupies a universal primary set without undergoing DNA sequencing phase in its real implementation (Ali *et al.*, 2004). In addition, RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar & Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari & Thakur, 2014), and is applicable for various laboratory situations (Kumar & Gurusubramanian, 2011). RAPD, therefore, was used in this current research in order to study genetic variation of various legumes, from *P. vulgaris* to *P. lunatus*.

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With respect to the aforementioned rationale, analyses of genetic diversity of *P. lunatus* in Indonesia are still of rarity. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in Africa (Fofana *et al.*, 2001), Europe (Sparvoli *et al.*, 2001), North America (Fofana *et al.*, 2001; Serrano *et al.*, 2010), Central America (Bi *et al.*, 2005) Latin America (Fofana *et al.*, 2001; Serrano *et al.*, 2010) and Caribbean countries (Fofana *et al.*, 2001). On one hand, researches in Indonesia are still limited to study on the potential of *P. lunatus* as alternative food source (Herry *et al.*, 2014; Diniyah *et al.*, 2013) alongside its essential substances (Tejasari, 2016; Nafi *et al.*, 2006; Diniyah *et al.*, 2015; Praseptiangga *et al.*, 2018; Sukatiningsih *et al.*, 2013). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti & Fauzi, 2019; Waluyo *et al.*, 2016). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on RAPD marker.

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**Commented [D12]:** In Indonesia, research about the potency of *P. lunatus* as alternative food source is limited

## MATERIAL AND METHODS

### Plant material

*P. lunatus* used in this research was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. On the basis of the result of identification in previous research (Purwanti & Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Each of the accessions, further, was planted in a polybag in which one polybag distanced one meter long to the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agent.

**Table 1.** List of accessions to analyze

### DNA Isolation

DNA isolation was administered based on CTAB method of Doyle & Doyle (1984), which was modified by Maftuchah & Zainuddin (2010). The used tissue was stemmed from leaf organ of 3-month-old plant. The followings were the procedures of DNA isolation: **1)** leaves were cut out into 2-3 pieces with the length of 1-2 cm; **2)** 0.3-gram leaves and liquid nitrogen were crushed using mortar and pestle; **3)** 0.002-gram Natrium bisulfite was weighed for each of 12 samples; **4)** 0.02-gram Na bisulfite was dissolved into 9,600- $\mu$ l buffer (labelled Buffer I); **5)** label was given to each 1.5-ml tube based on the name of the sample; **6)** Buffer I mixed with 800- $\mu$ l Natrium bisulfite was poured into each tube; **7)** the crushed sample was added to each tube; **8)** the tube was shaken until the crushed leaves were submersed into the buffer; **9)** the sample was incubated under the ideal temperature with the span of  $\pm 5$  minutes; **10)** the sample was centrifuged under the speed of 12.000 rpm, the temperature of 4°C with the span of 10 minutes; **11)** supernatant was eliminated; **12)** 500- $\mu$ l buffer II was added to the natant placed in the tube; **13)** the tube was vortexed; **14)** the sample was incubated under the temperature of 65°C with the span of 30 minutes while flipping over the tube for every 10 minutes during the incubation; **15)** chloroform was added: alcohol isoamyl (24:1) with the volume of  $\pm 500\mu$ l; **16)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **17)** the supernatant was taken and put into new tube; **18)** absolute ethanol with the volume of  $\pm 800\mu$ l was added along with natrium acetate with the volume of 80 $\mu$ l while doing homogenization through flipping over the tube all around; **19)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **20)** the supernatant was eliminated; **21)** 70%-concentrated ethanol with the volume of  $\pm 800 \mu$ l was added; **22)** the sample was centrifuged with the speed of 12,000 rpm under the temperature of 4°C during 10 minutes; **23)** the DNA pellet was dried out; **24)** Tris-EDTA

(TE) with the volume of 60 µl was added into tube; 25) the results of isolated DNA were stored under the temperature of -20°C.

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### PCR-RAPD

In the reaction of PCR, the isolated DNAs of *P. lunatus* L. were used as the mold. There were 10 kinds of primers used such as OPA6, OPA8, OPA10, OPA20, OPC19, OPD8, OPD12, OPE8, OPE15, and OPE16. The total volume of PCR reaction used signified 22.5 µl, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% gelatin); *dNTPS mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and molded DNA. The condition for PCR reaction was designed under pre-denaturation temperature of 94°C (in 5 minutes), denaturation temperature of 94°C (in 1 minute), primary attachment temperature of 36°C (in 1 minute), extension temperature of 72°C (in 2 minutes), post-extension temperature of 72°C (in 5 minutes), and post PCR reaction temperature of 4°C (in 2 minutes). For multiplication, the cycle of PCR reaction was repeated 36 times.

### Electrophoresis

There were three stages of procedure to confirm the result of isolation process as well as PCR reaction after implementation. The first stage was the creation of agarose gel with the concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labeled electrophoresis with the electrophoresis buffer of TBE (1x), loading dye (6x) under the condition of 60 V, 400 mA within 45 minutes. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

### Analysis on the DNA bands yielded from RAPD

Data analysis was performed by observing the pattern of visible bands from electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data indicating the existence and inexistence of bands typifying certain sizes. Afterwards, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and to create a phylogenetic tree by the assistance of Popgen software version 3.1.

## RESULTS AND DISCUSSION

In this current research, as many as 10 primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, the bands, as a result of PCR–RAPD amplification at those genotypes (originated from East Java), were assessed based on the binary data, with the description of: 1 for amplified band and 0 for unamplified. The following Table 2 shows the record of the number of locus found.

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**Table 2.** The record of the number of locus found at 15 genotypes of *Plunatus* L using 10 random primary

10 primers used to amplify the DNA at 15 genotypes of *P. lunatus* L. could reproduce DNA fragments with diverse sizes (polymorphic) despite its capability of yielding 68 DNA band lines (Table 3).

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**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.

Variation of genotype diversity at 15 genotypes resulted in 68 RAPD band assessed from 10 primary oligonucleotide with the means of 6.8 bands per primary. Out of the 10, 68 bands were yielded with various sizes of DNA fragments. The result of amplification showed that almost all bands indicated similar pattern of DNA band, polymorphic. The primary with the smallest number of bands was primary OPA10 and OPA20; while the one with the highest number of bands was OPD12. Moreover, there were 9 primers with 100% level of polymorphism. To be specific, OPC19 was only effective to result in 96% level of polymorphism.

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Next, cluster analysis upon the 68 RAPD bands was administered. This phase yielded a phylogenetic tree by the assistance of Neighbor-Joining method, equipped with similarity coefficient that ranged from 64%-100%, or there was genetic variation with the range of 0-36% (Figure 1). In genetic similarity coefficient of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with genetic similarity coefficient of 0.932 (including 8, 12, 13, 14 genotypes), A2 with genetic similarity coefficient of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). With respect to that with genetic similarity coefficient of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close. In addition, a phylogenetic tree constructed on the basis of RAPD analysis is shown in Figure 1.

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**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* under the study was relatively high (ranging from 96 to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Chacón-Sánchez & Martínez-Castillo, 2017; Camacho-Pérez, *et al.*, 2018), South America, and Afrika (Fofana, *et al.*, 1997). The existing high polymorphism had indicated the huge range of genetic diversity from the accessions under analysis.

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In nature, polymorphism constitutes one of a series of parameters to define genetic diversity on particular species (Snustad & Simmons, 2012). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of gene pool. According to previous study, the 15 accessions of *P. lunatus* included in current research were originated from 5 different gene pool, to name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti & Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The different ecological difference was closely interconnected with agroclimatic zone difference in which the origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

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The genetic diversity which was considered high had brought about an urgent implication in terms of crop improvement attempt, to include breeding procedure for quality improvement (Bhanu, 2017; Mohammadi & Prasanna, 2003; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not really effective as main food resource (Doria *et al.*, 2012; Offei *et al.*, 2003; Adeparusi, 2001). Consequently, the process of maturation in *P. lunatus* seeds was in need of proper and acceptable process, thus it could reduce the anti-nutritional substances (Adeparusi, 2001; Granito *et al.*, 2007; Sukatiningsih *et al.*, 2013). Further, by making use of data about genetic variety in breeding procedure, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factor. In addition, breeding procedure could be designed that way so as to yield the generation of *P. lunatus* with highly pest-resistant performance and shorter period of maturation phase.

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Next, with reference to the phylogenetic tree that was formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior.

Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width and pod width in comparison to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti & Fauzi, 2019). This demonstration was in line with phylogenetic tree resulted in this current research in which Probolinggo accessions as well as Accession 4 were clustered altogether in Cluster B.

Despite the fact, the result of cluster analysis also indicated the existence of accessions of which pod lengths were quite different, but still grouped in the same cluster. In contrast, when the pod length was not significantly different, the result of cluster analysis classified those accessions in different cluster. This was probably due to DNA related to RAPD marker used in this current research was unrelated to the character setting. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, moreover, the use of RAPD was not only affordable, but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. In spite of it, there were some studies of genetic variation on germplasm of beans which were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye & Ojobo, 2012), Turkey (Ince & Karaca, 2011), and in Vicia Faba, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez & Martínez-Castillo, 2017). In short, this current research has advocated the credibility of the use of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

## CONCLUSION

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analyzed. The result of RAPD utilizing 10 primers resulted in 68 bands in which 9 primers possessed 100% level of polymorphism and one primary was equipped with 96% level of polymorphism. On the basis of cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura

**Commented [D27]:** 1.Harap diganti atau diperbarui referensi yang dibawah tahun 2009.  
 2.Beberapa referensi yang in Indonesian, jika memungkinkan harap digantikan saja dengan yang in English  
 3.Beberapa references tidak dapat ditemukan secara online, jadi mohon semua references diberikan alamat URL nya

Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

**Table 2.** The record of the number of locus found at 15 genotypes of *P.lunatus* L using 10 random primary

Codes	Locus (bp)												
	10 0	20 0	30 0	40 0	50 0	60 0	70 0	80 0	90 0	100 0	110 0	120 0	1300
2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

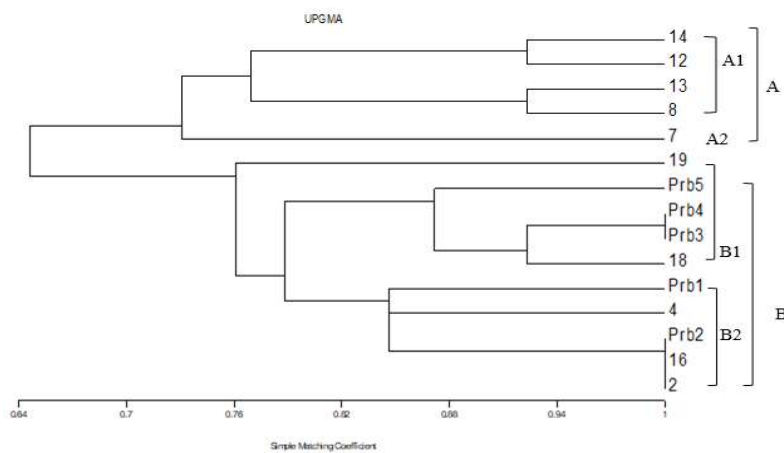
Description:

(1): DNA band was existent,

(0): DNA band was inexistent

**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.

No.	Markers (100 bp–2000 bp)	∑ Band	Polymorphic	% Polymorphic
1	OPA 6	8	8	100%
2	OPA 8	7	7	100%
3	OPA 10	5	5	100%
4	OPA 20	5	5	100%
5	OPC 19	6	5	96%
6	OPD 8	8	8	100%
7	OPD 12	9	9	100%
8	OPE 8	7	7	100%
9	OPE 15	7	7	100%
10	OPE 16	6	6	100%
Total		68	67	



**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker



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Diversity of *Phaseolus lunatus* L. in East Java based on Random Amplified Polymorphic DNA Marker

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**DIVERSITY OF *Phaseolus lunatus* L. IN EAST JAVA BASED ON RANDOM AMPLIFIED  
POLYMORPHIC DNA MARKER**

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**ABSTRACT**

*Phaseolus lunatus* L. is one of legume plants that can be found at some parts of Indonesia and is potential for alternative food rich of protein. This current research aimed at analyzing genetic accessions of *P. lunatus* distributed at some areas in East Java, Indonesia based on RAPD marker. A total of 15 accessions originated from four location were analyzed. In addition, there were 10 primers used, generating 68 lines of DNA band typified with different sizes. Further, there were 9 primers in the possession of 100% level of polymorphism in which only Primary OPC19 yielded 96% level of polymorphism. By means of Neighbor-Joining method, a phylogenetic tree was yielded by similarity coefficient of 64-100%. On the genetic similarity coefficient (GSC) of 0.6, there were two groups, the first and second major clusters, Cluster A and B. The former contained the accessions 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5. In

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conclusion, based on phylogenetic trees formed, *P. lunatus* from the same region tends to cluster in the same cluster.

**Keywords:** Genetic diversity, *Phaseolus lunatus*, phylogeny, RAPD.

## INTRODUCTION

*Phaseolus lunatus* L. is categorized as a legume plant with its great potential to become nutritious food. This kind of plant is also known as *koro* beans or lima beans and is identical with its vital nutritious contents, from protein (Tejasari, 2016) to fiber (Ancona *et al.*, 2011; Segura-Campos & Betancur-Ancona, 2010). In addition, this plant also contains extra nutrition stored at its stipule or stalk. In addition to its richness of nutritional contents, *P. lunatus* L. is also reported to contain a number of anti-oxidant compounds (Da et al., 2009) and other substances that are effective to optimize human's health (Da et al., 2009). For that reason, not only is *P. lunatus* L. known as protein-sourced food in many countries (Almeida & Pedrosa-Harand, 2011), it is also reported to be effective in preventing various illnesses (Da et al., 2009).

In addition to its benefits, *P. lunatus* L. can grow in some patterns of conditions (Lim, 2012). Therefore, it is common if *P. lunatus* can be found in many parts of countries as it has been domesticated there (Camacho-Pérez et al., 2018; Chacón-Sánchez & Martínez-Castillo, 2017; Félix et al., 2014). In Indonesia, to be specific, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti & Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions to exist in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as alternatively functional food source (Diniyah et al., 2013; Diniyah et al., 2015; Herry et al., 2013; Nafi et al., 2015). Now that Indonesia is dealing with serious issue of protein shortage in some particular areas (Diana et al., 2017; Ickowitz et al., 2016; Madanijah et al., 2016), maximizing the consumption of *P. lunatus* will be of effectiveness for the solution.

With respect to the attempt of elevating the use of *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content is in need of actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential foundation to run all the program of food source enhancement (Bhanu, 2017). Also, information that

pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a scheme of betterment for the species since the beginning of the period of systematic plant breeding (Bhanu, 2017). Furthermore, such kinds of information can be used to reach a phase of sustainable crop production (Fu, 2015). Moreover, research underpinning genetic diversity in particular plant also leads to an attempt of conservation (Carvalho et al., 2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using such numerous techniques as morphological identification, biochemical characterization, and analysis of DNA marker (Govindaraj et al., 2015). Related to the existence of those techniques, the selection of molecular marker is considered more appropriate and effective, not only to avoid any bias due to environmental influence but also to provide eclectic information related to genetic diversity in more acceptable way (Fu, 2015). There are some molecular markers included and considered particularly promising in helping analyse genetic diversity, namely RAPD, RFLP, and SCAR.

Amongst those markers, RAPD is defined as the most popular marker in many researches (Ben-Ari & Lavi, 2012). RAPD constitutes PCR-based technique which involves primary set with relatively short size and is able to randomly amplify many DNA segments (Kumari & Thakur, 2014). This technique is equipped with notable excellence in comparison with others, which is occupies a universal primary set without undergoing DNA sequencing phase in its real implementation. In addition, RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar & Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari & Thakur, 2014), and is applicable for various laboratory situations (Kumar & Gurusubramanian, 2011). Therefore, RAPD was often used as genetic marker in many researches that studying genetic variation of various legumes.

However, analyses of genetic diversity of *P. lunatus* in Indonesia are still of rarity. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in Africa (Fofana et al., 2001), Europe (Sparvoli et al., 2001), North America (Fofana et al., 2001; Serrano-Serrano et al., 2010), Central America (Bi et al., 2005), Latin America (Fofana et al., 2001; Serrano-Serrano et al., 2010) and Caribbean countries (Fofana et al., 2001). On one hand, in Indonesia, research about *P. lunatus* was still limited on its potency as alternative food source (Diniyah et al., 2013; Herry et al., 2014) alongside its essential substances (Diniyah et al., 2015; Praseptiangga et al., 2018; Sukatiningsih, Yustian, & Windarti, 2013; Tejasari, 2016). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti & Fauzi, 2019; Waluyo et al., 2016). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on RAPD marker.

## **MATERIAL AND METHODS**

### **Plant material**

*P. lunatus* used in this research was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. On the basis of the result of identification in previous research (Purwanti & Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Each of the accessions, further, was planted in a polybag in which one polybag distanced one meter long to the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agent.

**Table 1.** List of accessions to analyze

### **DNA Isolation**

DNA isolation was administered based on CTAB method of Doyle and Doyle (1984), which was modified by Maftuchah and Zainuddin (2010). The used tissue was stemmed from leaf organ of 3-month-old plant. Leaves were cut out and were crushed using liquid nitrogen. Then, Natrium bisulfited was weighed for each of 12 samples and dissolved into buffer. After the mixture was poured into each tube, the tube was incubated under the ideal temperature with the span of  $\pm 5$  minutes. After that, the supernatant produced after centrifugation was removed and buffer II was added. Then, chloroform was added and centrifuged again. Then, the supernatant was taken and put into new tube. Absolute ethanol was added along with natrium acetate and the sample was centrifuged again. After the supernatant was eliminated; 70%-concentrated ethanol was added and the sample was centrifuged again. After that, the DNA pellet was dried out. Finally, Tris-EDTA (TE) was added into tube and the results of isolated DNA were stored under the temperature of  $-20^{\circ}\text{C}$ .

### **PCR-RAPD**

In the reaction of PCR, the isolated DNAs of *P. lunatus* L. were used as the mold. There were 10 kinds of primers used such as OPA6, OPA8, OPA10, OPA20, OPC19, OPD8, OPD12, OPE8, OPE15, and OPE16. The total volume of PCR reaction used signified 22.5  $\mu\text{l}$ , containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM  $\text{MgCl}_2$ ; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche);  $\text{dH}_2\text{O}$ ; and molded DNA. The condition for PCR reaction was designed under pre-denaturation temperature of  $94^{\circ}\text{C}$  (in 5 minutes), denaturation temperature of  $94^{\circ}\text{C}$  (in 1 minute), primary attachment temperature of  $36^{\circ}\text{C}$  (in 1 minute), extension temperature of  $72^{\circ}\text{C}$  (in 2 minutes), post-extension temperature of  $72^{\circ}\text{C}$  (in 5 minutes), and post PCR reaction temperature of  $4^{\circ}\text{C}$  (in 2 minutes). For multiplication, the cycle of PCR reaction was repeated 36 times.

### **Electrophoresis**

There were three stages of procedure to confirm the result of isolation process as well as PCR reaction after implementation. The first stage was the creation of agarose gel with the concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labeled electrophoresis with the electrophoresis buffer of TBE (1x), loading dye (6x) under the condition of 60 V, 400 mA within 45 minutes. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

#### **Analysis on the DNA bands yielded from RAPD**

Data analysis was performed by observing the pattern of visible bands from electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data indicating the existence and inexistence of bands typifying certain sizes. Afterwards, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and to create a phylogenetic tree by the assistance of Popgen software version 3.1.

### **RESULTS AND DISCUSSION**

In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, the bands, as a result of PCR–RAPD amplification at those genotypes (originated from East Java), were assessed based on the binary data, with the description of: 1 for amplified band and 0 for unamplified. The following Table 2 showed the record of the number of locus found.

**Table 2.** The record of the number of locus found at 15 genotypes of *P.lunatus* L using 10 random primary

After PCR was conducted, DNA fragments with diverse sizes (polymorphic) were produced. Total, 68 DNA band lines was yielded (Table 3).

**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.

Variation of genotype diversity at 15 genotypes resulted in 68 RAPD band assessed from 10 primary oligonucleotide with the means of 6.8 bands per primary. The result of amplification showed that almost all bands indicated similar pattern of DNA band, polymorphic. The primer with the smallest number of bands was OPA10 and OPA20; while the one with the highest number of bands was OPD12.

Moreover, there were 9 primers with 100% level of polymorphism. To be specific, OPC19 was only effective to result in 96% level of polymorphism.

Next, cluster analysis upon the 68 RAPD bands was administered. This phase yielded a phylogenetic tree by the assistance of Neighbor-Joining method, equipped with similarity coefficient that ranged from 64%-100%, or there was genetic variation with the range of 0-36% (Figure 1). In GSC of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with GSC of 0.932 (including 8, 12, 13, 14 genotypes), A2 with GSC of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). At GSC of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close. In addition, a phylogenetic tree constructed on the basis of RAPD analysis is shown in Figure 1.

**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* was relatively high (ranging from 96 to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Camacho-Pérez et al., 2018; Chacón-Sánchez & Martínez-Castillo, 2017). The existing high polymorphism had indicated the huge range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes one of a series of parameters to define genetic diversity on particular species (Snustad & Simmons, 2012). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of gene pool. According to previous study, the 15 accessions of *P. lunatus* included in current research were originated from 5 different gene pool, to name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti & Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang et al., 2016). The ecological condition was closely

interconnected with agroclimatic zone which it was grown. In the origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity which was considered high had brought about an urgent implication in terms of crop improvement attempt, to include breeding procedure for quality improvement (Bhanu, 2017; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not really effective as main food resource (Doria et al., 2012). Consequently, the process of maturation in *P. lunatus* seeds was in need of proper and acceptable process, thus it could reduce the anti-nutritional substances (Sukatiningsih et al., 2013). Further, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factor. In addition, breeding procedure could be designed that way so as to yield the generation of *P. lunatus* with highly pest-resistant performance and shorter period of maturation phase.

Next, with reference to the phylogenetic tree that was formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior. Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width and pod width in comparison to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti & Fauzi, 2019).

Despite the fact, the result of cluster analysis also indicated the existence of accessions of which pod lengths were quite different, but still grouped in the same cluster. In contrast, when the pod length was not significantly different, the result of cluster analysis classified those accessions in different cluster. This was probably due to DNA related to RAPD marker used in this current research was unrelated to the character setting. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, moreover, the use of RAPD was not only affordable, but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. In spite of it, there were some studies of genetic variation on germplasm of beans which were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari et al., 2015), South Africa (Adesoye & Ojobo, 2012), Turkey (Ince & Karaca, 2011), and in *Vicia Faba*, Palestine (Basheer-Salimia et al., 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic



variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez et al., 2018) and SNP (Chacón-Sánchez & Martínez-Castillo, 2017). In short, this current research has advocated the credibility of the use of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

## CONCLUSION

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analyzed. The result of RAPD utilizing 10 primers resulted in 68 bands in which 9 primers possessed 100% level of polymorphism and one primary was equipped with 96% level of polymorphism. On the basis of cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Table 1.** List of accessions to analyze

<b>Accession Codes</b>	<b>Origins</b>
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura
Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

**Table 2.** The record of the number of locus found at 15 genotypes of *Plunatus* L using 10 random primary

<b>Codes</b>	<b>Locus (bp)</b>												
	10	20	30	400	50	60	70	80	90	10	110	12	1300
	0	0	0		0	0	0	0	0	00	0	00	

2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

Description:

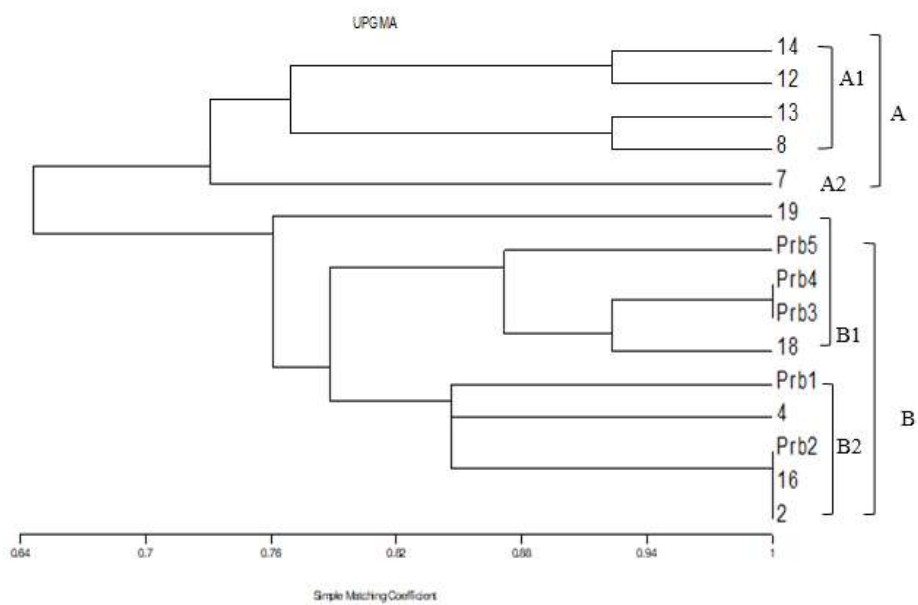
(1): DNA band was existent,

(0): DNA band was inexistent

**Table 3.** RAPD primers used for diversity analysis of *Phaseolus lunatus* L.

No.	Markers (100 bp–2000 bp)	$\Sigma$ Band	Polymorphic	% Polymorphic
1	OPA 6	8	8	100%
2	OPA 8	7	7	100%
3	OPA 10	5	5	100%
4	OPA 20	5	5	100%
5	OPC 19	6	5	96%

6	OPD 8	8	8	100%
7	OPD 12	9	9	100%
8	OPE 8	7	7	100%
9	OPE 15	7	7	100%
10	OPE 16	6	6	100%
Total		68	67	



**Fig. 1.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker



**Pengumuman pengalihan jurnal target (dari MAB ke JJBS) (5 Oktober 2020)**



## **ICON-BEAT 2019**

**International Conference on Bioenergy and Environmentally Sustainable Agriculture Technology 2019**  
GKB 4, 9th Floor, University of Muhammadiyah Malang, 7 November 2019 (Conference) and 8 November 2019 (Workshop Clinic)  
Website: <http://icon-beat.umm.ac.id>  
Email: [icon-beat@umm.ac.id](mailto:icon-beat@umm.ac.id)

---

October 5<sup>th</sup>, 2020

Dear author,

I am pleased to inform you that your submitted paper to The International Conference on Bio-Energy and Environmentally Sustainable Agriculture Technologies (ICoN- BEAT) that has been presented on 7<sup>th</sup> – 8<sup>th</sup> November 2019 under the title below has been selected to publish in the Jordan Journal of Biological Science (JJBS) (url: <http://jjbs.hu.edu.jo/>; url scimago: <https://www.scimagojr.com/journalsearch.php?q=21100385604&tip=sid>).

ID No. : ABS 119

Title : Genetic Diversity of Lima Beans (*Phaseolus lunatus* L.) in East Java, Indonesia Based on Random Amplified Polymorphic DNA Marker

The change from Malaysian Applied Biology Journal (MAB) to JJBS due to scientific journal ranking of MAB has downgrade from Q3 to the Q4. Therefore we would like to apologize for this inconvenience.

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Thank you

Warmest regards,

A handwritten signature in blue ink, appearing to read 'Listiari', with a stylized flourish at the end.

Dr. Ir. Listiari Hendraningsih, MP.  
ICON-BEAT 2019 Chairperson

## Pengisian copyright form dan cover letter dari JJBS (31 Maret 2021)



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**Corresponding author:** Ahmad Fauzi

To be published in the journal: Jordan Journal of Biological Sciences (JJBS)

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## Cover Letter

**Manuscript title:** Diversity of *Phaseolus lunatus* L. in East Java, Indonesia based on Random Amplified Polymorphic DNA Marker

**Research Subject Area:** Biotechnology

**Authorships [Full Name for all Authors] and Affiliation:**

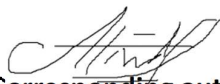
- 1- Elly Purwanti, Department of Biology Education, Faculty of Teacher Training and Education, University of Muhammadiyah Malang, Indonesia.
- 2- Mohamad Amin, Department of Biology, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, Indonesia
- 3- Siti Zubaidah, Department of Biology, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, Indonesia
- 4- Maftuchah Maftuchah, Department of Agrotechnology, Faculty of Agriculture and Animal Science, University of Muhammadiyah Malang, Indonesia
- 5- Siti Nur Hidayati, Department of Biology, Middle Tennessee State University, USA
6. Ahmad Fauzi, Department of Biology Education, Faculty of Teacher Training and Education, University of Muhammadiyah Malang, Indonesia

**Conflict of interest/sponsorship (if any):**

**Ethical committee approval \ Human Research Protections \ or Institutional Review Board (IRB):** If Applicable, Please provide a copy of the Approval.

**We affirm that the submission represents work that has not been published previously and is not currently being considered by another journal. Also, I confirm that each author has seen and approved the contents of the submitted manuscript.**

**Signature (on behalf of all co-authors (if any))**



**Corresponding author**

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**Submission date:** March 30, 2021

Permintaan revisi dari JJBS (16 April 2021)

Manuscript Evaluation Report- Referee1

Manuscript ID: JJBS 36/21/R6 Due date: April 15, 2021

TITLE:

Diversity of Phaseolus lunatus L. in East Java, Indonesia based on Random  
Amplified Polymorphic DNA MarkerType of Article:  Review Article  Research Paper  Case Report**PART A:**

On a scale of 1 – 5 (1 being lowest and 5 being highest), rate the manuscript based on the following criteria;

NO.	Criteria	Score
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3	Are the keywords and abstract sufficient and informative?	2
4	What is the scholarly quality of the manuscript?	1
5	Is this a new and/ or original contribution?	2
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7	Are the methods of data analysis acceptable?	2
8	Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?	2
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10	Are the interpretations/ conclusions sound and justified by the data?	2
11	Are the References in a proper format according to JJBS author Instruction?	
12	Is the MS written in correct and satisfactory English?	1

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3

**PART B: Comments per Section of Manuscript:**

Abstract	
Introduction	

Methodology	
Results	
Discussion and Conclusion	
References	

**PART C: Recommendation ( Kindly Mark With An ✓ )**

Acceptable in its Present Form	
Acceptable with Minor Revision	
Reconsidered after Major Revision	
Reject on Ground of ( Please Be Specific)	As it is (Reject) However, after substantial language editing, new resubmission

**PART D: Additional Comments:**

Please add any other additional comments or specific suggestions on the enclosed comments sheet:

**The manuscript needs substantial language editing. I started to do editing, but the whole manuscript is hard to follow  
I recommend editing the manuscript and resubmitted it as a new submission**

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Deanship of Scientific Research

TITLE:

Diversity of Phaseolus lunatus L. in East Java, Indonesia based on Random Amplified Polymorphic DNA Marker

Type of Article:  Review Article  Research Paper  Case Report

**PART A:**

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7	Are the methods of data analysis acceptable?	3
8	Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?	3
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7

**PART B: Comments per Section of Manuscript:**

<b>Abstract</b>	Add unique band results to your abstract...
<b>Introduction</b>	Very long...
<b>Methodology</b>	PCR condition and amount of material used not clear...
<b>Results</b>	Add agarose gel figure of DNA extraction and PCR amplification of all primers...
<b>Discussion and Conclusion</b>	Add unique band results to your dis. And conclusion..

<b>References</b>	Write it according to the journal require....

**PART C: Recommendation ( Kindly Mark With An ✓ )**

<b>Acceptable in its Present Form</b>	
<b>Acceptable with Minor Revision</b>	
<b>Reconsidered after Major Revision</b>	✓
<b>Reject on Ground of ( Please be Specific)</b>	

**PART D: Additional Comments:**

Please add any other additional comments or specific suggestions on the enclosed comments sheet:

Please make all necessary correction....Best regards.

**Pengiriman naskah revisi ke JJBS (24 April 2021)**

<b>Section</b>	<b>Reviewer Comments</b>	<b>Author Responses</b>
<b>Abstract</b>	Add unique band results to your abstract...	We have added information related to the unique band that we get
<b>Introduction</b>	Very long...	We have reduced our introduction
<b>Methodology</b>	PCR condition and amount of material used not clear...	We have detailed the methods we used in our study
<b>Results</b>	Add agarose gel figure of DNA extraction and PCR amplification of all primers...	We have inserted a photo of the amplified result we obtained for ten primers we used
<b>Discussion and Conclusion</b>	Add unique band results to your dis. And conclusion..	We have added information regarding the number of unique bands, the length of the bands, as well as a description of the accessions where the bands appear
<b>References</b>	Write it according to the journal require....	We have adjusted the reference format of our manuscript with the JJBS author guideline



Title:

Diversity of *Phaseolus lunatus* L. in East Java, Indonesia based on PCR-RAPD technique

Manuscript: 36/21/R6

**Keywords:**

Genetic diversity, Lima bean, *phylogeny*, RAPD

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**DIVERSITY OF *Phaseolus lunatus* L. IN EAST JAVA INDONESIA BASED ON PCR-RAPD  
TECHNIQUE**

**ABSTRACT**

*Phaseolus lunatus* L. is one of the legume plants found in some parts of Indonesia and is potential for alternative food rich in protein. This current research aimed at analysing genetic accessions of *P. lunatus* distributed at some areas in East Java, Indonesia, based on the RAPD marker. A total of 15 accessions

originated from four locations were analysed. Ten primers used and produced 68 bands, out of 67 were polymorphic. The percent polymorphism was 96 to 100%. Ten unique bands were detected in eight accessions (Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6). Using the Neighbor-Joining method, a phylogenetic tree was yielded by a similarity coefficient of 64 % to 100 %. On the genetic similarity coefficient (GSC) of 0.6, there were two clusters, the first and second major clusters, Cluster A and B. The former contained the accessions 7, 8, 13, and 14, while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5. In conclusion, based on phylogenetic trees formed, *P. lunatus* from the same region tend to cluster in the same cluster.

**Keywords:** Genetic diversity; Lima bean Phylogeny, RAPD-PCR.

## 1. Introduction

*Phaseolus lunatus* L. is categorized as a legume plant with its great potential to become nutritious food. In Indonesia, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti and Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions exist in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as an alternatively functional food source (Diniyah *et al.*, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2013; Nafi *et al.*, 2015). Nowadays, Indonesia is dealing with the severe issue of protein shortage in some particular areas (Diana *et al.*, 2017; Ickowitz *et al.*, 2016; Madanijah *et al.*, 2016), maximizing the consumption of *P. lunatus* will be of effectiveness for the solution.

With respect to elevating the use of elevating *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content needs actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential foundation to run all the program of food source enhancement (Bhanu, 2017). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a betterment scheme for the species since the beginning of the period of systematic plant

breeding (Bhanu, 2017). Furthermore, such information can be used to reach a phase of sustainable crop production (Fu, 2015). Moreover, research underpinning genetic diversity in a particular plant also leads to conservation (Carvalho *et al.*, 2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using numerous techniques such as morphological identification, biochemical characterization, and analysis of **molecular** markers (Govindaraj *et al.*, 2015). Related to those techniques, the selection of molecular markers is considered more appropriate and effective, not only to avoid any bias due to environmental influence but also to provide eclectic information related to genetic diversity in a more acceptable way (Fu, 2015). Some molecular markers are included and considered particularly promising in helping analyse genetic diversity, **such as** RAPD, RFLP, and SCAR.

Among those markers, RAPD is the most popular marker in many researches (AIRawashdeh and AIRawashdeh, 2015; Ben-Ari and Lavi, 2012). RAPD constitutes a PCR-based technique that involves a primary set with a relatively short size and can be a PCR-based technique that involves a relatively short size and can randomly amplify many DNA segments (Kumari and Thakur, 2014). This technique is equipped with outstanding excellence compared to others, which occupies a universal primary set without undergoing the DNA sequencing phase in its real implementation. In addition, the RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar and Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari and Thakur, 2014), and is applicable for various laboratory situations (Kumar and Gurusubramanian, 2011). Therefore, RAPD was often used as a genetic marker in many research the genetic variation of various legumes.

However, analyses of the genetic diversity of *P. lunatus* in Indonesia are still of rarity. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in North America (Serrano-serrano *et al.*, 2010), Central America (Camacho-Pérez *et al.*, 2018), and South America (Silva *et al.*, 2019). On the one hand, in Indonesia, research about *P. lunatus* was still limited on its potency as an alternative food source (Diniyah *et al.*, 2013; Herry *et al.*, 2014) alongside its essential substances (Diniyah *et al.*, 2015; Praseptiangga *et al.*, 2018; Sukatiningsih *et al.*, 2013; Tejasari, 2016). In addition,

researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti and Fauzi, 2019). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on the RAPD marker.

## 2. Material and Methods

### 2.1. Collection of Samples

*P. lunatus* used in this present study was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. On the basis of the result of identification in previous research (Purwanti and Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Further, each of the accessions, planted in a polybag in which one polybag distanced one meter long to the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agent.

**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura

19	Madura
Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

## 2.2. DNA Isolation

DNA isolation was administered based on CTAB method of Doyle and Doyle (1984), which was modified by Maftuchah and Zainuddin (2010). The used tissue was stemmed from the leaf organ of 3 **month-old** plants. Leaves were cut out and were crushed using liquid nitrogen. Then, Natrium bisulfited was weighed for each of 12 samples and dissolved into the buffer. The results of isolated DNA were stored under the temperature of -20 °C.

**Table 2.** List of primers

<b>Primers</b>	<b>Sequence</b>	<b>GC Content (%)</b>
OPA6	GGTCCCTGAC	70
OPA8	GTGACGTAGG	60
OPA10	GTGATCGCAG	60
OPA20	GTTGCGATCC	60
OPC19	GTTGCCAGCC	70
OPD8	GTGTGCCCCA	70
OPD12	CACCGTATCC	60
OPE8	TCACCACGGT	60
OPE15	ACGCACAACC	60

OPE16	GGTGACTGTG	60
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### 2.3. PCR-RAPD

Ten primers having 60-70 GC content were used in this present study (Table 2). The total volume of PCR reaction used signified 22.5  $\mu$ L, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01 % gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and 30 ng DNA template. The condition for PCR reaction was designed under pre-denaturation temperature of 94 °C (in 5 min), denaturation temperature of 94 °C (in 1 min), primary attachment temperature of 36 °C (in 1 min), extension temperature of 72 °C (in 2 min), post-extension temperature of 72 °C (in 5 min), and post PCR reaction temperature of 4 °C (in 2 min). For multiplication, the cycle of PCR reaction was repeated 36 times.

### 2.4. Agarose Gel Electrophoresis

There were three stages of procedure to confirm the result of isolation process as well as PCR reaction after implementation. The first stage was the creation of agarose gel with the concentration of 0.8 % (for isolation result) and 1 % (for PCR result) as a medium of running DNA. Next, the stage was labeled electrophoresis with the electrophoresis buffer of TBE (1 $\times$ ), loading dye (6 $\times$ ) under the condition of 60 V, 400 mA within 45 min. At last, the stage was the coloration using 10 %-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

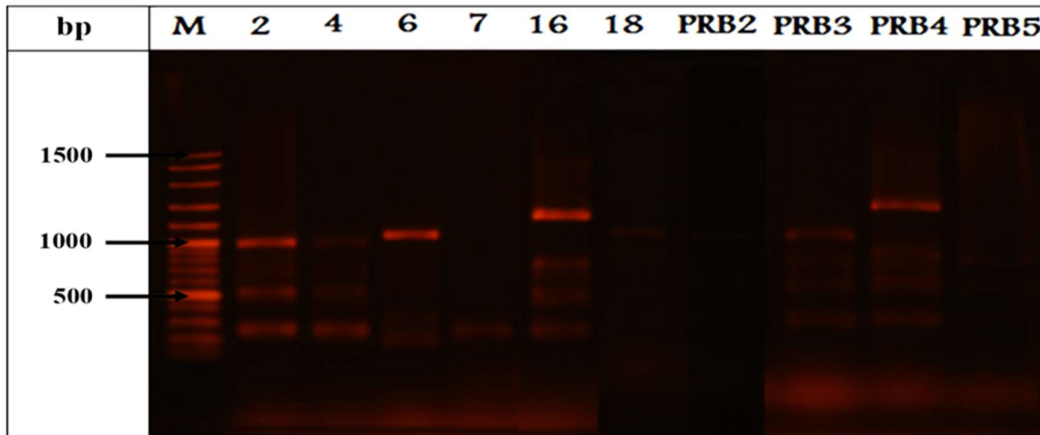
### 2.5. Analysis on the DNA Bands Yielded from RAPD

Data analysis was performed by observing the pattern of visible bands from electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data (0 and 1) indicating the existence and inexistence of bands typifying certain sizes. Afterwards, the existing bands were observed

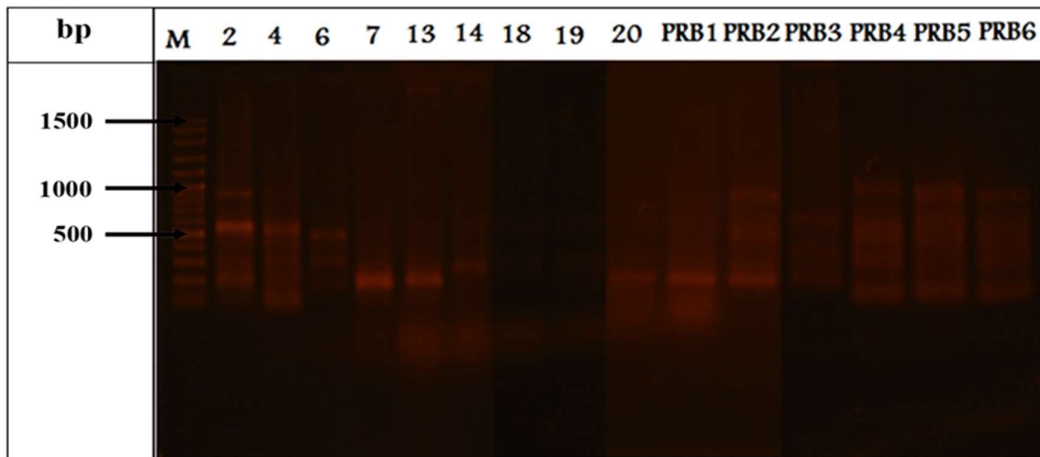
to identify the percentage of polymorphic and monomorphic bands and to create a phylogenetic tree by the assistance of Popgen software version 3.1 (Yeh and Boyle, 1997).

### 3. Results and Discussion

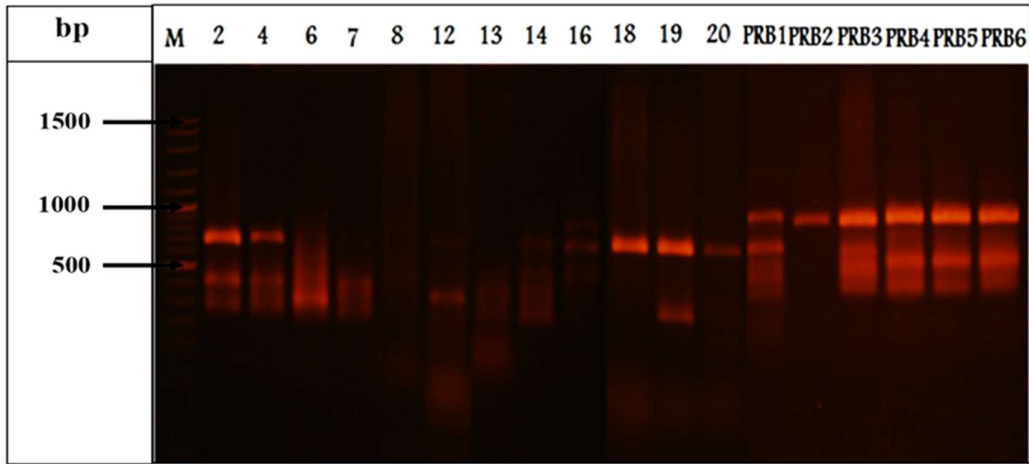
In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, the bands, as a result of PCR–RAPD amplification at those genotypes (Figure 1), were assessed based on the binary data, with the description of: 1 for amplified band and 0 for unamplified. The following Table 3 showed the record of the number of locus found.



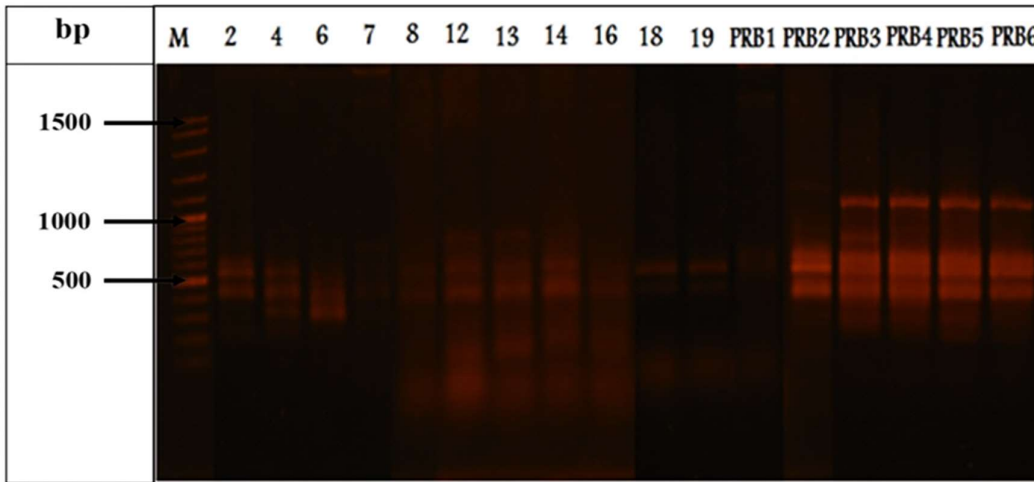
(a)



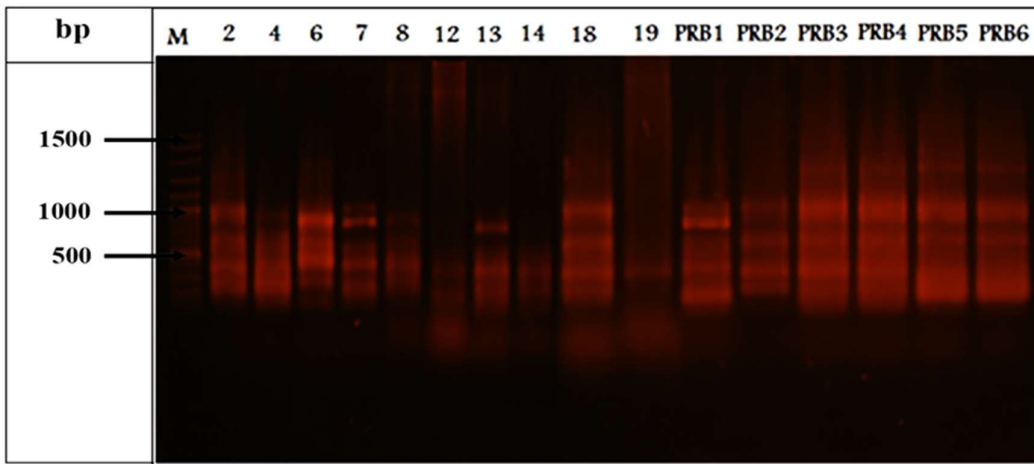
(b)



(c)

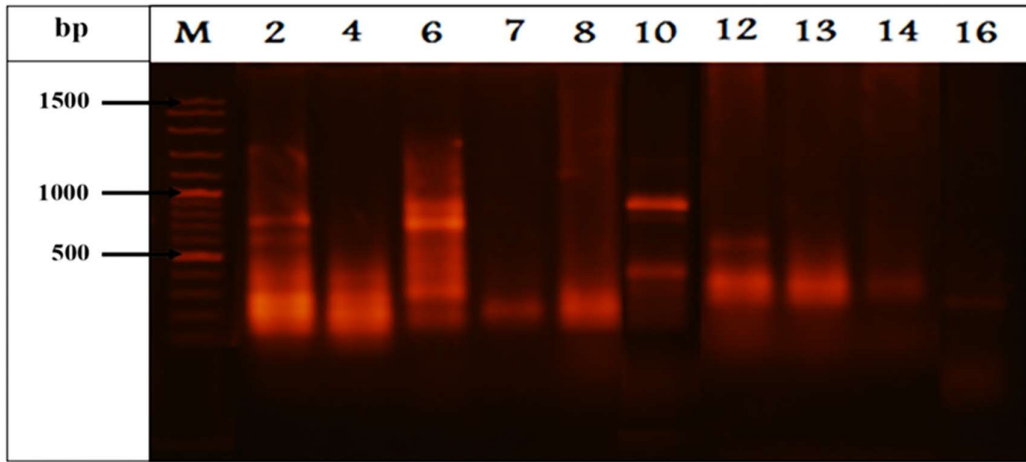


(d)

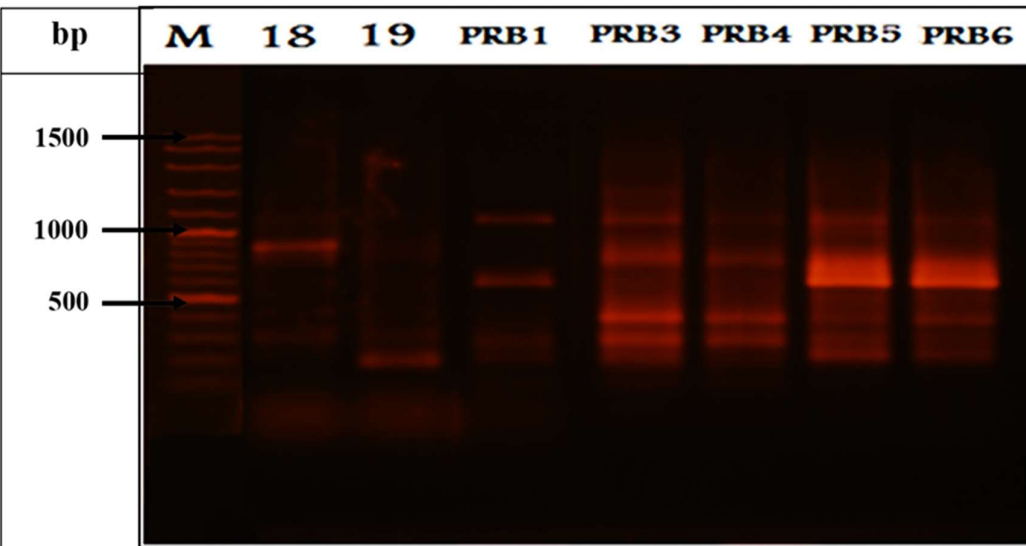
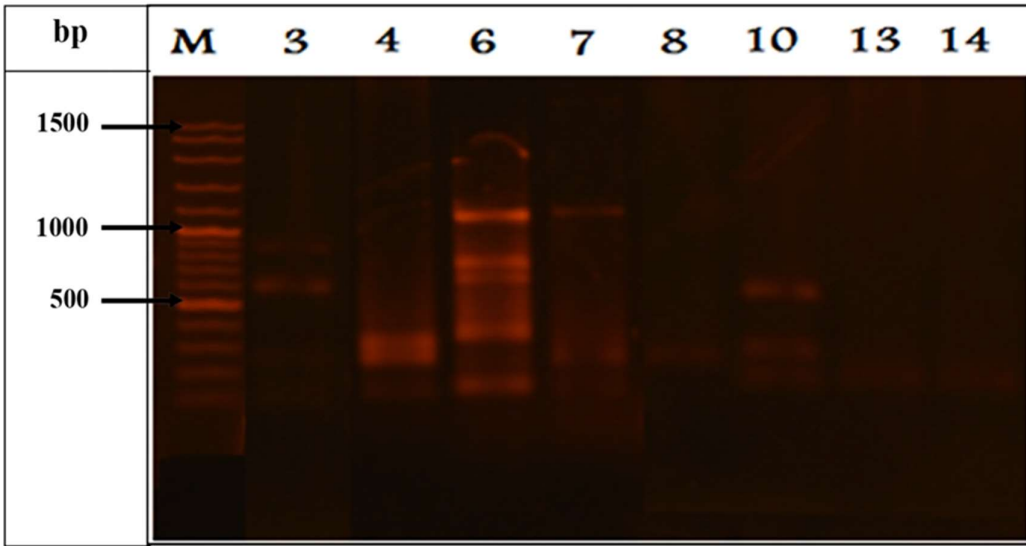


(e)

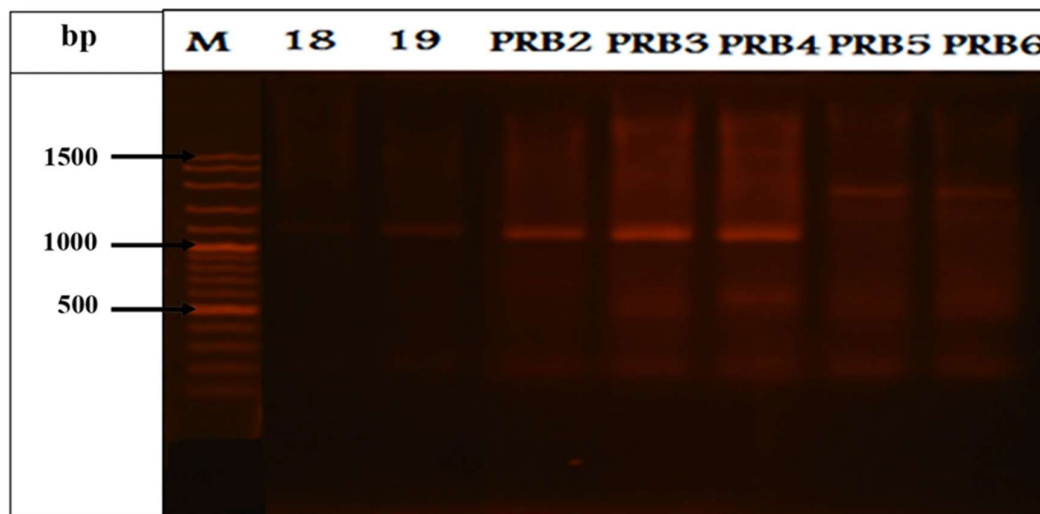
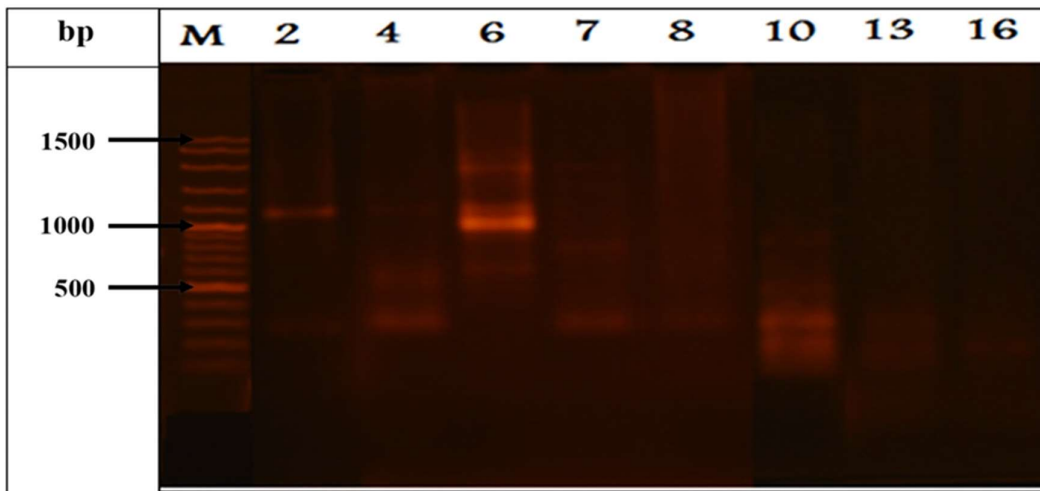




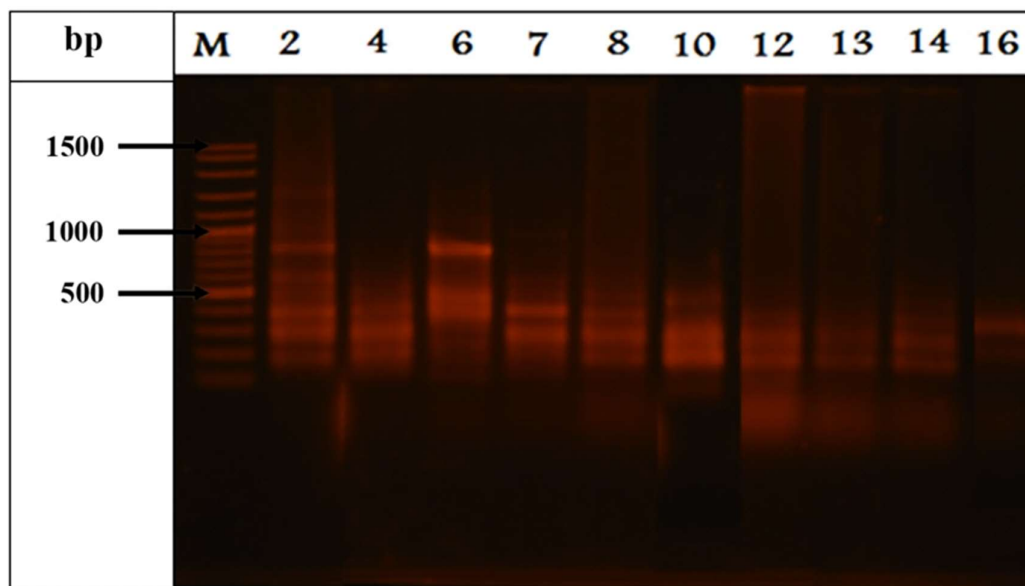
(f)

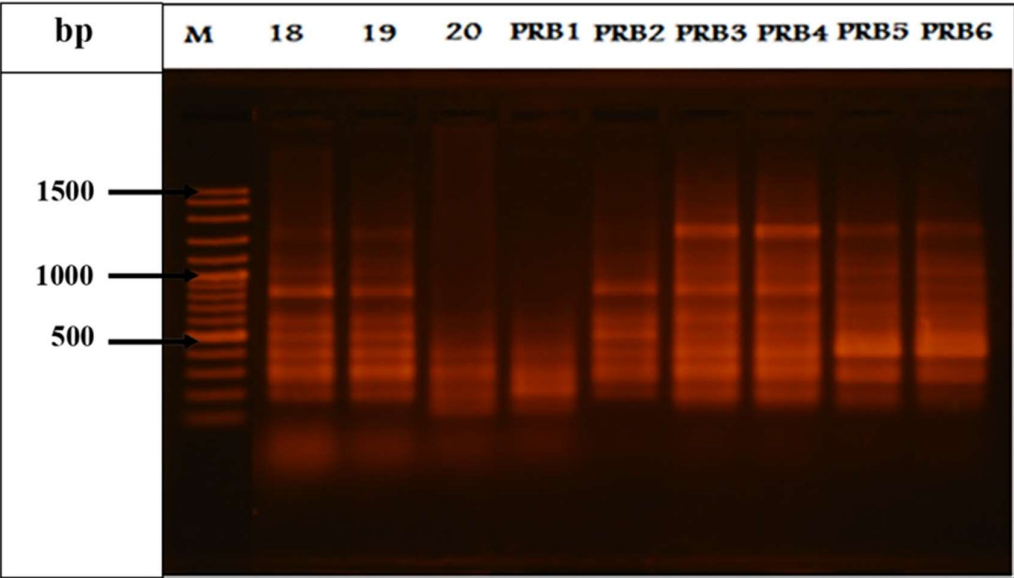


(g)

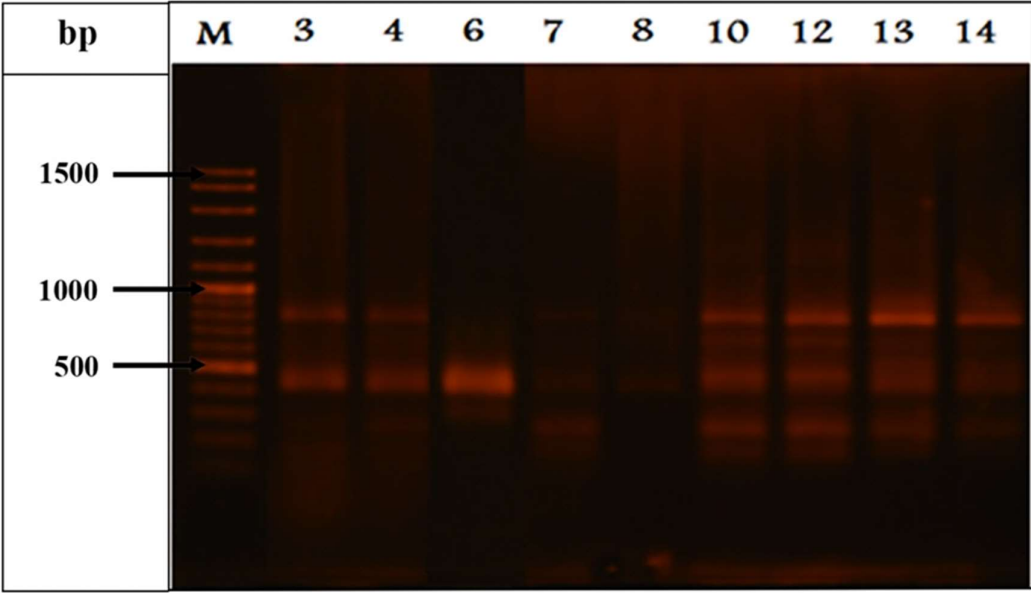


(h)





(i)





<b>18</b>	1	1	1	1	1	1	1	1	1	0	1	1	0
<b>19</b>	0	1	1	1	1	1	1	1	0	0	1	1	0
<b>Prb1</b>	1	1	1	1	1	1	1	0	1	1	1	0	0
<b>Prb2</b>	0	1	1	1	1	1	1	1	1	1	1	0	0
<b>Prb3</b>	1	1	1	1	1	1	1	1	1	1	1	1	0
<b>Prb4</b>	1	1	1	1	1	1	1	1	1	1	1	1	0
<b>Prb5</b>	1	1	1	1	1	1	1	1	1	0	1	1	1

Description:

(1): DNA band was existent,

(0): DNA band was inexistent

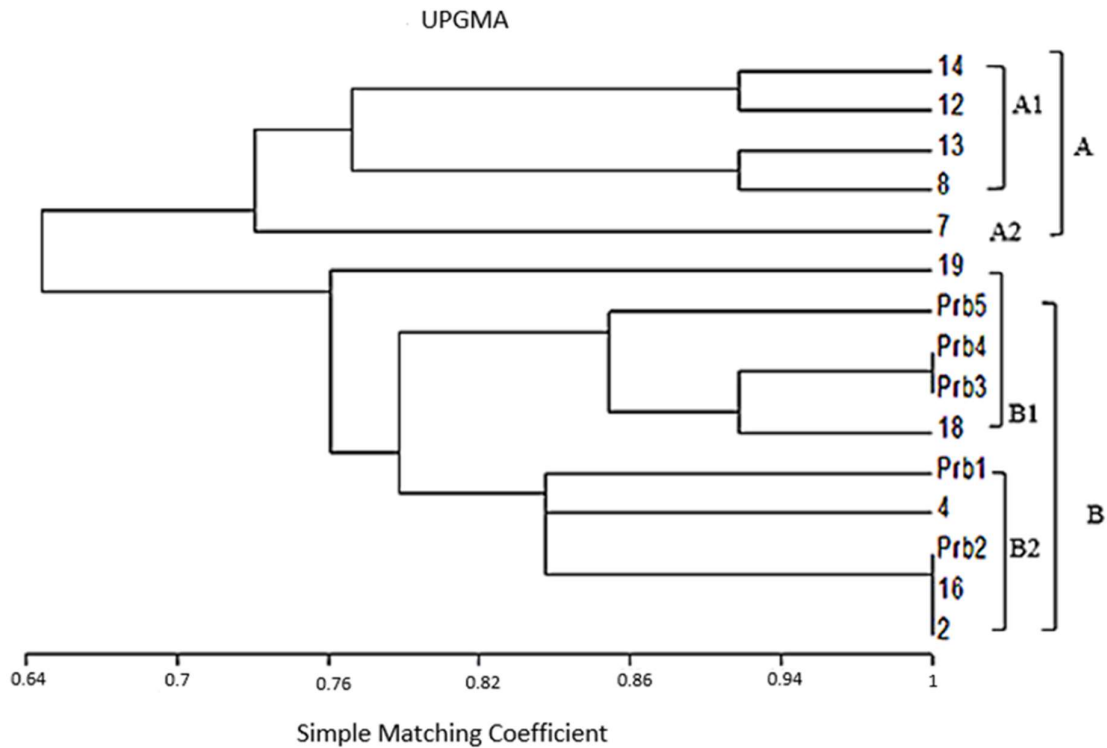
After PCR was conducted, DNA fragments with diverse sizes (polymorphic) were produced (Table 4). A total of 68 amplified bands were obtained, out of 67 were polymorphic. The percent polymorphism was 96 to 100%. The total number of amplified bands varied between 5 (OPA10 and OPA20) to 9 (OPA12) with an average of 6,8 bands per primer. Ten unique bands were detected in several accessions. The size of unique band ranged from 200 (Mdr6) to 1300 bp (Prb5).

**Table 4.** RAPD primers used for diversity analysis of *P. lunatus*.

No.	Markers (100 bp– 2000 bp)	$\Sigma$ Band	Polymorphic	% Polymorphic	Unique band		
					Total	Locus	Accession
1	OPA 6	8	8	100 %	2	700	Mdr16
						200	Mdr6
2	OPA 8	7	7	100 %	0	-	-
3	OPA 10	5	5	100 %	0	-	-

4	OPA 20	5	5	100 %	1	300	Mdr4
5	OPC 19	6	5	96 %	0	-	-
6	OPD 8	8	8	100 %	1	500	Prb1
7	OPD 12	9	9	100 %	2	1200	Prb3
8	OPE 8	7	7	100 %	2	1300	Prb5
9	OPE 15	7	7	100 %	0	-	-
10	OPE 16	6	6	100 %	2	700	Mdr12
						500	Mdr16
	Total	68	67		10		

The cluster analysis upon the 68 RAPD bands was administered. **The phylogenetic tree using Neighbor-Joining method was produced**, equipped with similarity coefficient that ranged from 64 % to 100 %, or there was genetic variation with the range of 0 % to 36 % (Figure 2). In GSC of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with GSC of 0.932 (including 8, 12, 13, 14 genotypes), A2 with GSC of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). At GSC of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close.



**Fig. 2.** The phylogenetic tree showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* was relatively high (ranging from 96 % to 100 %). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Camacho-Pérez *et al.*, 2018; Chacón-Sánchez and Martínez-Castillo, 2017). The existing high polymorphism had indicated the huge range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes one of a series of parameters to define genetic diversity on particular species (Singh and Kulathinal, 2013). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of gene pool. According to previous study, the 15 accessions of *P. lunatus* included in current research were originated from five different gene pool, to name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti and Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The ecological condition was closely interconnected with agroclimatic zone which it was grown. In the origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity which was considered high had brought about an urgent implication in terms of crop improvement attempt, to include breeding procedure for quality improvement (Bhanu, 2017; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not really effective as main food resource (Doria *et al.*, 2012). Consequently, the process of maturation in *P. lunatus* seeds was in need of proper and acceptable process, thus it could reduce the anti-nutritional substances (Sukatarningsih *et al.*, 2013). Further, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factor. In addition, breeding procedure could be designed that way so as to yield the generation of *P. lunatus* with highly pest-resistant performance and shorter period of maturation phase.

Next, with reference to the phylogenetic tree that was formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior. Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width and pod width in comparison to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti and Fauzi, 2019).



Despite the fact, the result of cluster analysis also indicated the existence of accessions of which pod lengths were quite different, but still grouped in the same cluster. In contrast, when the pod length was not significantly different, the result of cluster analysis classified those accessions in different cluster. This was probably due to DNA related to RAPD marker used in this current research was unrelated to the character setting. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, the use of RAPD was not only affordable, but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. In spite of it, there were some studies of genetic variation on germplasm of beans which were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye and Ojobo, 2012), Turkey (Ince and Karaca, 2011), and in *Vicia Faba*, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez and Martínez-Castillo, 2017). In short, this current research has advocated the credibility of the use of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

#### **4. Conclusion**

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analysed. The result of RAPD utilizing ten primers resulted in 68 bands in which nine primers possessed 100 % level of polymorphism and one primary was equipped with 96 % level of polymorphism. Ten unique bands were detected in eight accessions, i.e. Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6. On the basis of cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13 and 14; while the latter comprised the accessions of 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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# Diversity of *Phaseolus lunatus* L. in East Java, Indonesia based on PCR-RAPD technique

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## Abstract

*Phaseolus lunatus* L. is one of the legume plants found in some parts of Indonesia and has potential as alternative food rich in protein. This current research aimed at analysing genetic accessions of *P. lunatus* distributed in some areas in East Java, Indonesia, based on the RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) marker. 15 accessions originated from four locations were analysed. Ten primers were used and produced 68 bands out of 67 were polymorphic. The percent polymorphism was 96% to 100%. Ten unique bands were detected in eight accessions (Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6). Using the Neighbor-Joining method, a phylogenetic tree was yielded by a similarity coefficient of 64% to 100%. On the genetic similarity coefficient (GSC) of 0.6, there were two clusters: the first and second major clusters (Cluster A and B). The former contained the accessions 7, 8, 13, and 14, while the latter comprised 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5. In conclusion, based on phylogenetic trees formed, *P. lunatus* from the same region cluster in the same cluster.

**Keywords:** Genetic diversity; Lima bean, Phylogeny, Polymerase Chain Reaction, Random Amplified Polymorphic Deoxyribonucleic acid

## 1. Introduction

*Phaseolus lunatus* L. is categorized as a legume plant with great potential to become nutritious food. In Indonesia, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti and Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions existing in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as an alternatively functional food source (Diniyah *et al.*, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2013; Nafi *et al.*, 2015). Nowadays, Indonesia is dealing with the severe issue of protein shortage in some areas (Diana *et al.*, 2017; Ickowitz *et al.*, 2016; Madaniyah *et al.*, 2016), so maximizing consumer consumption *P. lunatus* will be an effective solution.

With respect to elevating *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content needs actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential foundation to run all the programs of food source enhancement (Bhanu, 2017). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a betterment scheme for the species since the beginning of systematic plant breeding (Bhanu, 2017). Furthermore, such information can be used to reach a phase of sustainable crop production (Fu, 2015). Moreover, the research underpinning genetic diversity in a particular plant also leads to conservation (Carvalho *et al.*,

2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using numerous techniques such as morphological identification, biochemical characterization, and analysis of molecular markers (Govindaraj *et al.*, 2015). Related to those techniques, the selection of molecular markers is considered more appropriate and effective to avoid any bias due to environmental influence and provide eclectic information about genetic diversity in a more acceptable way (Fu, 2015). Some molecular markers are included and considered particularly promising in helping analyse genetic diversity, such as RAPD, RFLP, and SCAR.

Among those markers, RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) is the most popular marker in many research projects (AlRawashdeh and AlRawashdeh, 2015; Ben-Ari and Lavi, 2012). RAPD constitutes a PCR-based (Polymerase Chain Reaction) technique that involves a primary set with a relatively short size and can be a PCR-based technique that involves a relatively short size and can randomly amplify many DNA segments (Kumari and Thakur, 2014). This technique is equipped with outstanding excellence compared to others, which occupies a universal primary set without undergoing the DNA sequencing phase in its actual implementation. In addition, the RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar and Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari and Thakur, 2014), and is applicable for various laboratory situations (Kumar and Gurusubramanian, 2011). Therefore, RAPD has often been

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used as a genetic marker in much research on the genetic variation of various legumes.

However, analyses of the genetic diversity of *P. lunatus* in Indonesia are still rare. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in North America (Serrano-serrano *et al.*, 2010), Central America (Camacho-Pérez *et al.*, 2018), and South America (Silva *et al.*, 2019). On the one hand, in Indonesia, research about *P. lunatus* was still limited on its potency as an alternative food source (Diniyah *et al.*, 2013; Herry *et al.*, 2014) alongside its essential substances (Diniyah *et al.*, 2015; Praseptianga *et al.*, 2018; Sukatiningsih *et al.*, 2013; Tejasari, 2016). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti and Fauzi, 2019). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on the RAPD marker.

## 2. Material and Methods

### 2.1. Collection of Samples

*P. lunatus* used in this present study was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. Based on the result of identification in previous research (Purwanti and Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Further, each of the accessions was planted in a polybag in which one polybag was distanced one meter long from the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agents.

**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura
Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

### 2.2. DNA Isolation

DNA isolation was administered based on the CTAB method of Doyle and Doyle (1984), which was modified by Maftuchah and Zainuddin (2010). The used tissue stemmed from the leaf organ of 3 mo (month-old) plants. First, leaves were cut out and were crushed using liquid nitrogen. Then, Natrium bisulfited was weighed for each of 12 samples and dissolved into the buffer. The results of isolated DNA were stored under a temperature of -20 °C.

**Table 2.** List of primers

Primers	Sequence 5'-3'	GC Content (%)
OPA6	GGTCCCTGAC	70
OPA8	GTGACGTAGG	60
OPA10	GTGATCGCAG	60
OPA20	GTTGCGATCC	60
OPC19	GTTGCCAGCC	70
OPD8	GTGTGCCCCA	70
OPD12	CACCGTATCC	60
OPE8	TCACCACGGT	60
OPE15	ACGCACAACC	60
OPE16	GGTGACTGTG	60

### 2.3. PCR-RAPD

Ten primers having 60 to 70 GC content were used in this present study (Table 2). The total volume of PCR reaction used signified 22.5 µL, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and 30 ng DNA template. The condition for PCR reaction was designed under the pre-denaturation temperature of 94 °C (in 5 min), denaturation temperature of 94 °C (in 1 min), primary attachment temperature of 36 °C (in 1 min), extension temperature of 72 °C (in 2 min), the post-extension temperature of 72 °C (in 5 min), and post PCR reaction temperature of 4 °C (in 2 min). For multiplication, the cycle of the PCR reaction was repeated 36 times.

### 2.4. Agarose Gel Electrophoresis

There were three stages of procedure to confirm the result of isolation process and PCR reaction after implementation. The first stage was creating agarose gel with a concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labelled electrophoresis with the electrophoresis buffer of TBE (1×), loading dye (6×) under the condition of 60 V, 400 mA within 45 min. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

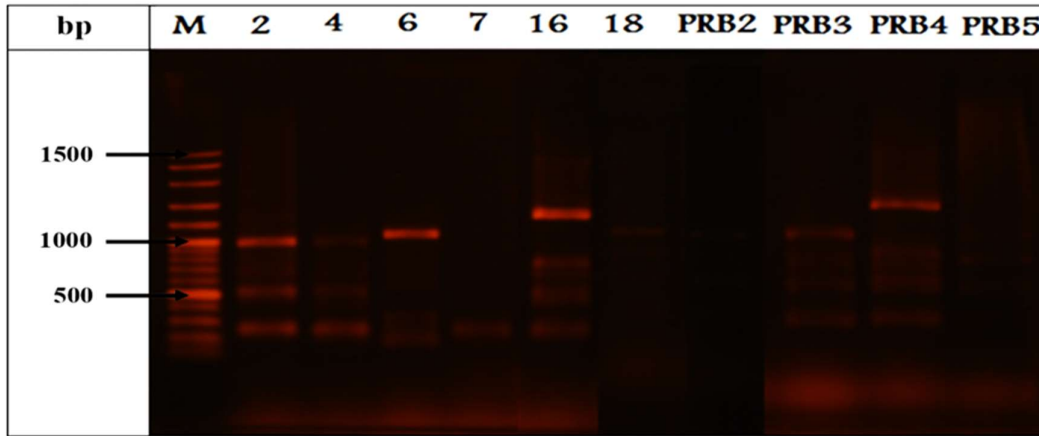
### 2.5. Analysis on the DNA Bands Yielded from RAPD

Data analysis was performed by observing the pattern of visible bands from the electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data (0 and 1), indicating the existence and inexistence of bands typifying specific sizes. Afterward, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and create a phylogenetic tree by creating a phylogenetic tree with Popgen software version 3.1 (Yeh and Boyle, 1997).

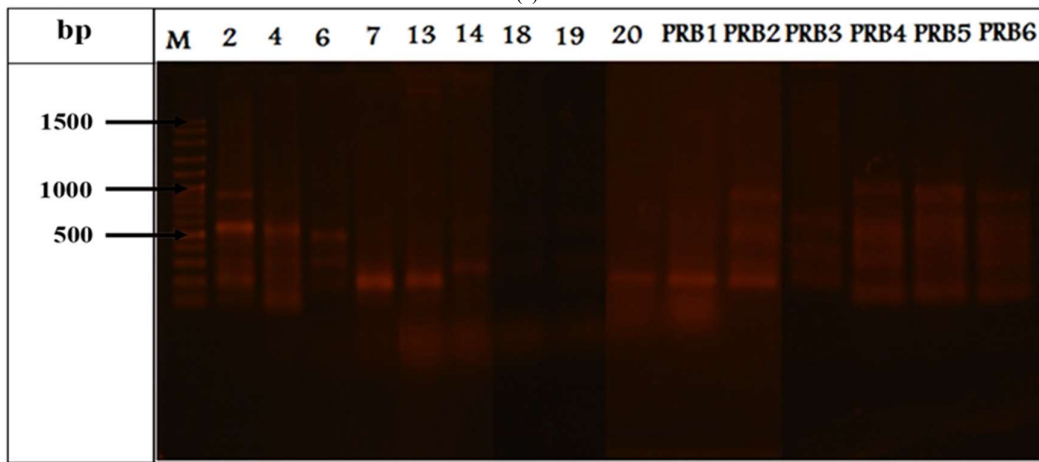
### 3. Results and Discussion

In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, as a result of PCR-RAPD amplification at those genotypes

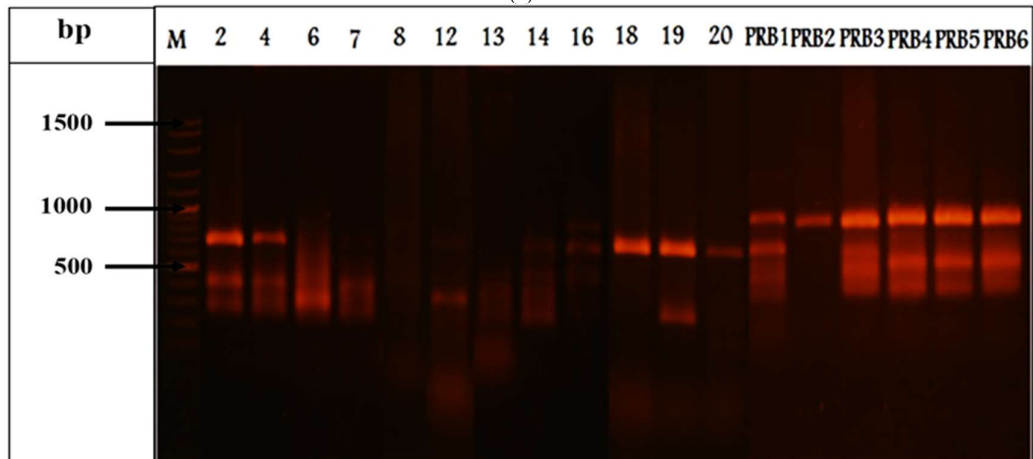
(Figure 1), the bands were assessed based on the binary data, with the description of 1 for the amplified band and 0 for unamplified. The following Table 3 showed the record of the number of loci found.



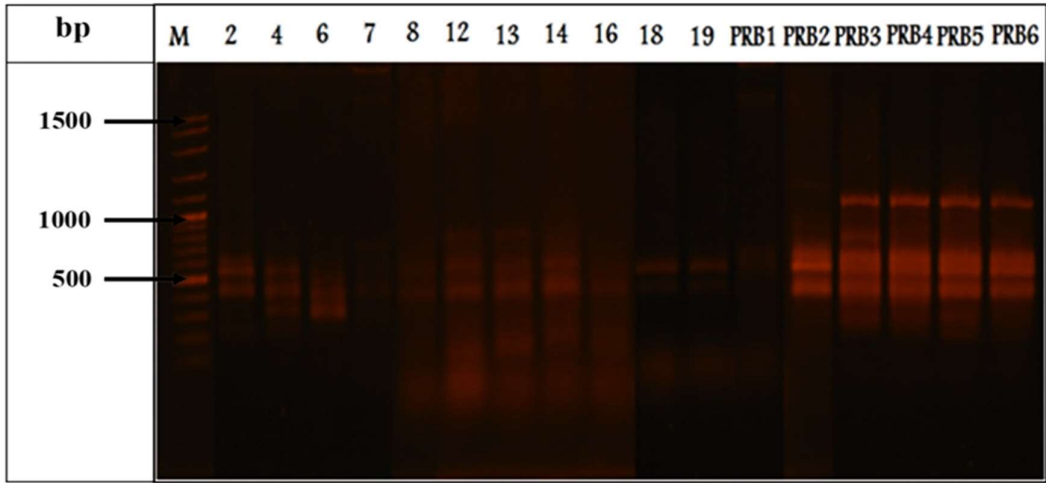
(a)



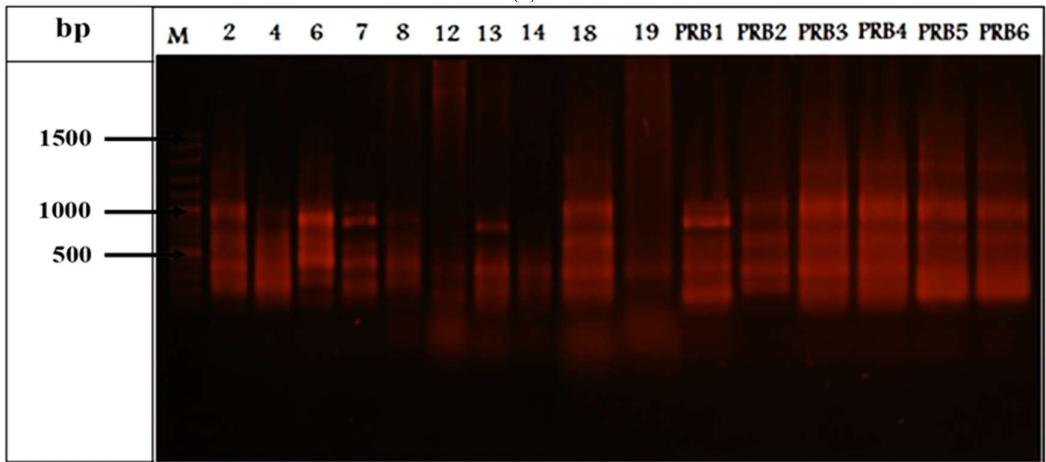
(b)



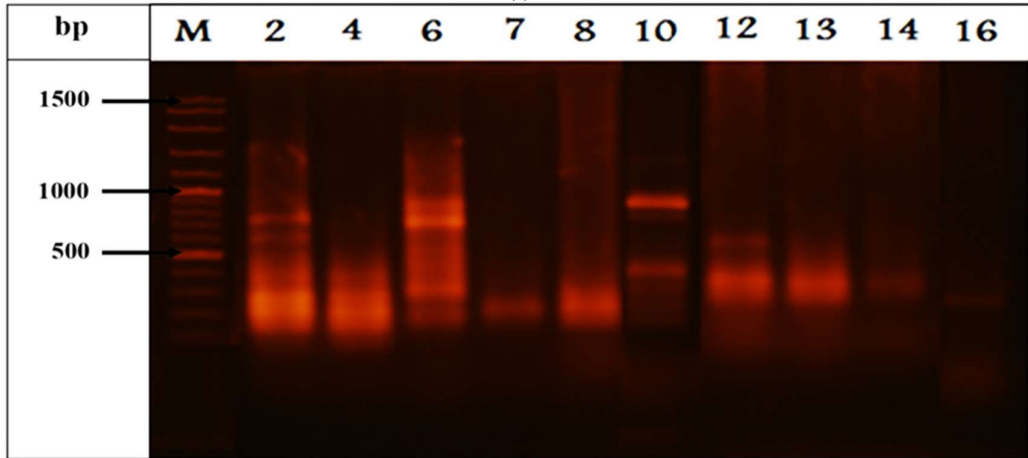
(c)



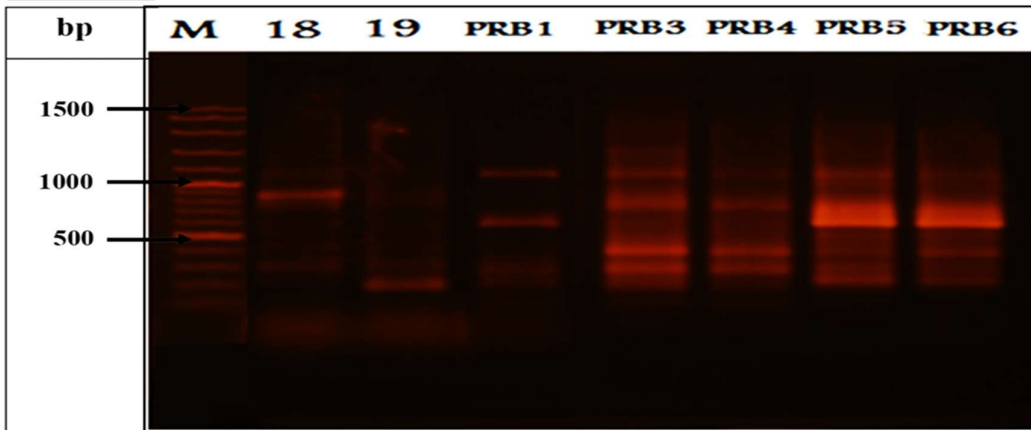
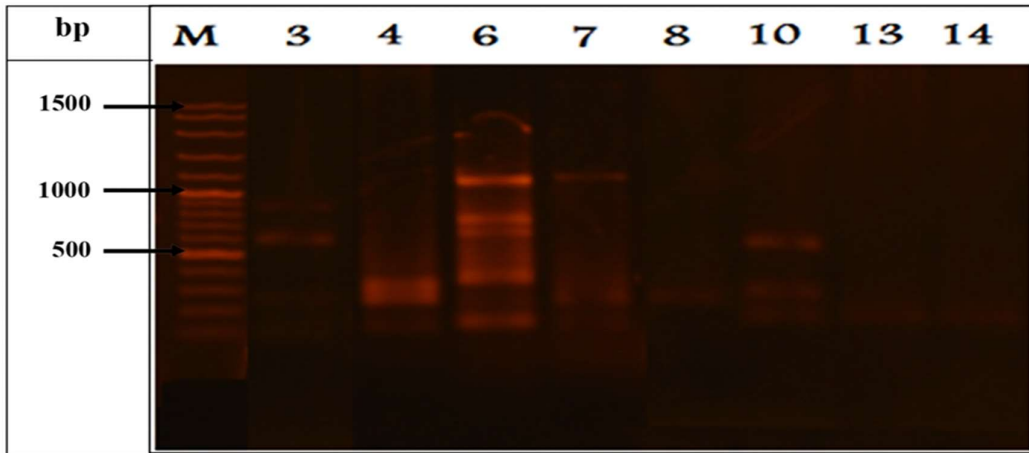
(d)



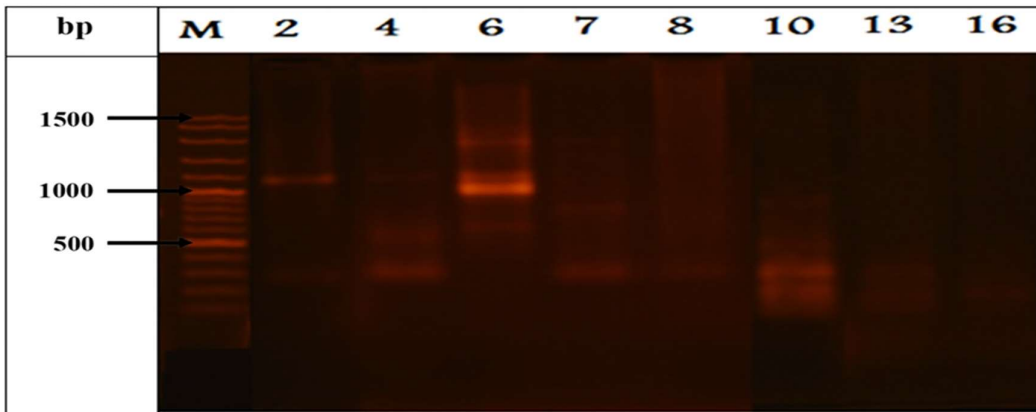
(e)



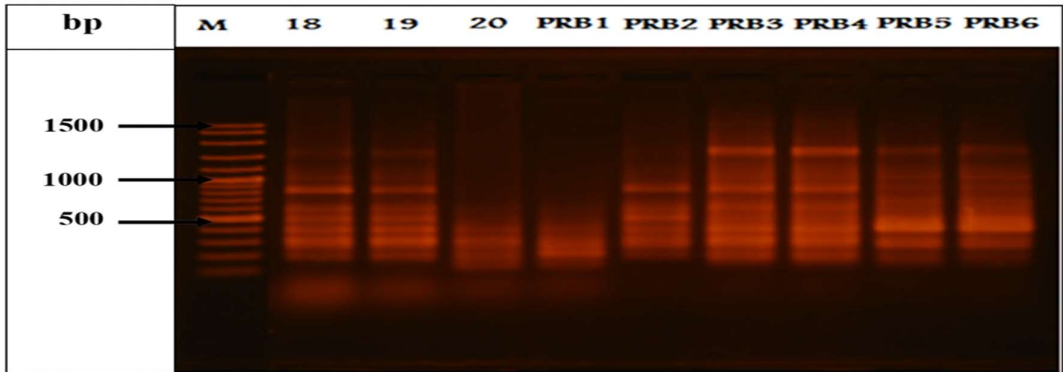
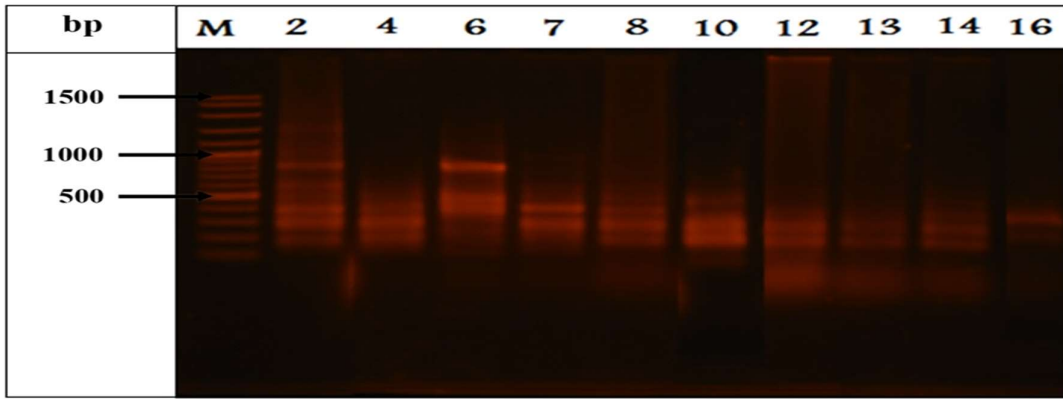
(f)



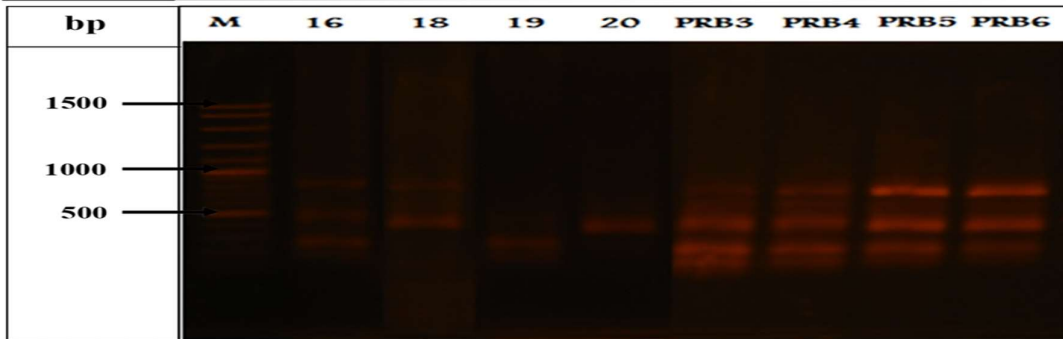
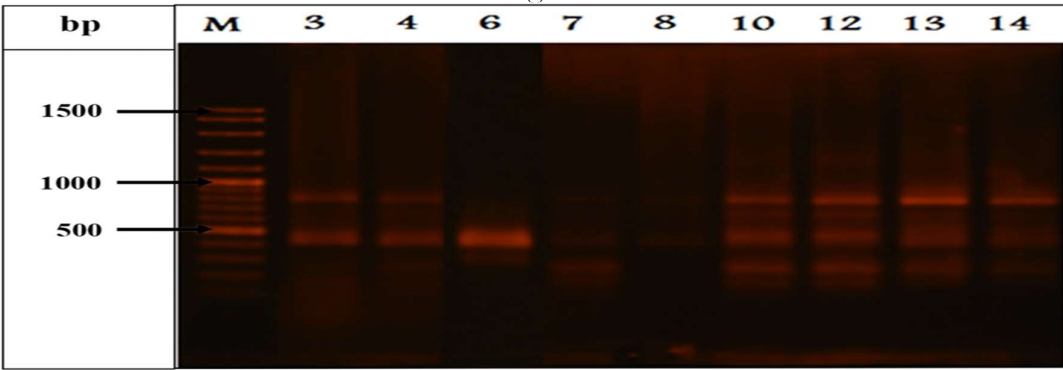
(g)



(h)



(i)



(j)

Figure 1. Gel electrophoresis profiles for PCR-RAPD results using (a) OPA6, (b) OPA8, (c) OPA10, (d) OPA20, (e) OPA19, (f) OPD8, (g) OPD12, (h) OPE8, (i) OPE15, (j) OPE16. M is the DNA size ladder

**Table 3.** The record of the number of locus found at 15 genotypes of *P. lunatus* using 10 random primary

Codes	Locus (bp)												
	100	200	300	400	500	600	700	800	900	1000	1100	1200	1300
2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

Description: (1): DNA band was existent, (0): DNA band was non-existent.

After PCR was conducted, DNA fragments of diverse sizes (polymorphic) were produced (Table 4). A total of 68 amplified bands were obtained, out of 67 were polymorphic. The percent polymorphism was 96% to 100%. The total number of amplified bands varied between 5 (OPA10 and

OPA20) to 9 (OPA12), with an average of 6.8 bands per primer. Ten unique bands were detected in several accessions. The size of the unique band ranged from 200 (Mdr6) to 1 300 bp (Prb5).

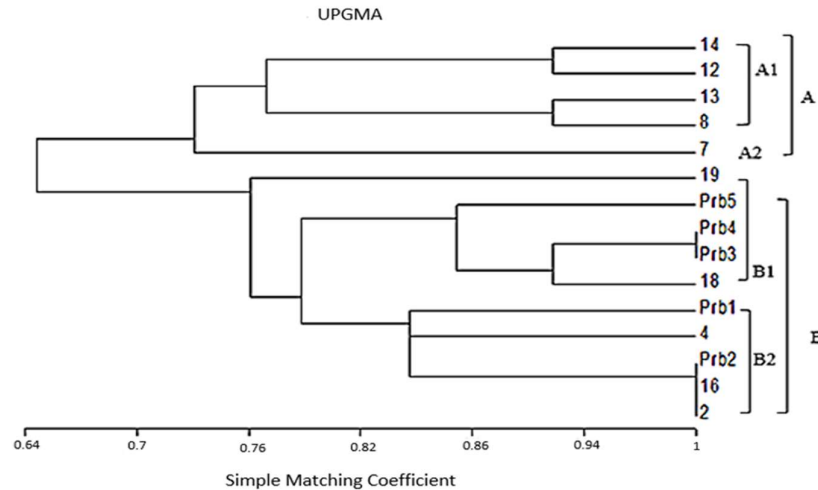
Table 4. RAPD primers used for diversity analysis of *P. lunatus*.

No.	Markers (100 bp– 2000 bp)	Σ Band	Polymorphic	% Polymorphic	Unique band		
					Total	Locus	Accession
1	OPA 6	8	8	100%	2	700 200	Mdr16 Mdr6
2	OPA 8	7	7	100%	0	-	-
3	OPA 10	5	5	100%	0	-	-
4	OPA 20	5	5	100%	1	300	Mdr4
5	OPC 19	6	5	96%	0	-	-
6	OPD 8	8	8	100%	1	500	Prb1
7	OPD 12	9	9	100%	2	1 200 900	Prb3 Mdr19
8	OPE 8	7	7	100%	2	1 300 500	Prb5 Mdr4
9	OPE 15	7	7	100%	0	-	-
10	OPE 16	6	6	100%	2	700 500	Mdr12 Mdr16
Total		68	67		10		

The cluster analysis upon the 68 RAPD bands was administered. The phylogenetic tree using Neighbor-Joining method was produced, equipped with similarity coefficient that ranged from 64% to 100%, or there was genetic variation with the range of 0% to 36% (Figure 2). In GSC of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with GSC of

0.932 (including 8, 12, 13, 14 genotypes), A2 with GSC of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). At GSC of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close.





**Figure 2.** The dendrogram showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* was relatively high (ranging from 96% to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Camacho-Pérez *et al.*, 2018; Chacón-Sánchez and Martínez-Castillo, 2017). The existing high polymorphism had indicated the vast range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes a series of parameters to define genetic diversity on particular species (Singh and Kulathinal, 2013). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of the gene pool. According to the previous study, the 15 accessions of *P. lunatus* included in the current research were originated from five different gene pools, name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti and Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The ecological condition was closely interconnected with the agro-climatic zone in which it was grown. The origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity considered high had brought about an urgent implication in terms of crop improvement attempt, including the breeding procedure for quality improvement (Bhanu, 2017; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not effective as the main food resource (Doria *et al.*, 2012). Consequently, the process of maturation in *P. lunatus* seeds needed proper and acceptable process; thus it could reduce the anti-nutritional substances (Sukatningsih *et al.*, 2013). Further, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factors. In addition, the breeding procedure could be designed that way to yield the generation of *P.*

*lunatus* with highly pest-resistant performance and a shorter period of maturation phase.

Next, concerning the phylogenetic tree formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior. Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width, and pod width compared to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti and Fauzi, 2019).

Despite this fact, cluster analysis also indicated the existence of accessions of which pod lengths were quite different but still grouped in the same cluster. In contrast, when the pod length was not significantly different, cluster analysis classified those accessions in different clusters. This was probably due to DNA related to the RAPD marker used in this current research being unrelated to the character set. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, the use of RAPD was not only affordable but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. However, there were some studies of genetic variation on germplasm of beans that were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye and Ojobo, 2012), Turkey (Ince and Karaca, 2011), and in Vicia Faba, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez and Martínez-Castillo, 2017). In



short, this current research has advocated the credibility of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

#### 4. Conclusion

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analysed. The result of RAPD using ten primers resulted in 68 bands in which nine primers possessed 100% level of polymorphism, and one primary was equipped with 96% level of polymorphism. In addition, ten unique bands were detected in eight accessions, i.e. Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6. Based on cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13, and 14, while the latter comprised the 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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**Pengiriman hasil koreksi (16 Mei 2021)**

<b>Section</b>	<b>Notes</b>	<b>Changes</b>
<b>Authors name</b>	The fourth author's name is written twice	Maftuchah Maftuchah becomes Maftuchah
<b>Authors affiliation</b>	Our university name needs to be changed in Indonesian	University of Muhammadiyah Malang becomes Universitas Muhammadiyah Malang
<b>Abstract</b>	None	None
<b>Introduction</b>	None	None
<b>Methodology</b>	None	None
<b>Results</b>	None	None
<b>Discussion and Conclusion</b>	None	None
<b>References</b>	The Editor said "Please omit the Doi no. from the list of references"	We've removed DOIs from every reference

# Diversity of *Phaseolus lunatus* L. in East Java, Indonesia based on PCR-RAPD technique

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## Abstract

*Phaseolus lunatus* L. is one of the legume plants found in some parts of Indonesia and has potential as alternative food rich in protein. This current research aimed at analysing genetic accessions of *P. lunatus* distributed in some areas in East Java, Indonesia, based on the RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) marker. 15 accessions originated from four locations were analysed. Ten primers were used and produced 68 bands out of 67 were polymorphic. The percent polymorphism was 96% to 100%. Ten unique bands were detected in eight accessions (Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6). Using the Neighbor-Joining method, a phylogenetic tree was yielded by a similarity coefficient of 64% to 100%. On the genetic similarity coefficient (GSC) of 0.6, there were two clusters: the first and second major clusters (Cluster A and B). The former contained the accessions 7, 8, 13, and 14, while the latter comprised 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5. In conclusion, based on phylogenetic trees formed, *P. lunatus* from the same region cluster in the same cluster.

**Keywords:** Genetic diversity; Lima bean, Phylogeny, Polymerase Chain Reaction, Random Amplified Polymorphic Deoxyribonucleic acid

## 5. Introduction

*Phaseolus lunatus* L. is categorized as a legume plant with great potential to become nutritious food. In Indonesia, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti and Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions existing in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as an alternatively functional food source (Diniyah *et al.*, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2013; Nafi *et al.*, 2015). Nowadays, Indonesia is dealing with the severe issue of protein shortage in some areas (Diana *et al.*, 2017; Ickowitz *et al.*, 2016; Madaniyah *et al.*, 2016), so maximizing consumer consumption *P. lunatus* will be an effective solution.

With respect to elevating *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content needs actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential foundation to run all the programs of food source enhancement (Bhanu, 2017). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a betterment scheme for the species since the beginning of systematic plant breeding (Bhanu, 2017). Furthermore, such information can be used to reach a phase of sustainable crop production (Fu, 2015). Moreover, the research underpinning genetic diversity in a particular plant also leads to conservation (Carvalho *et al.*,

2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using numerous techniques such as morphological identification, biochemical characterization, and analysis of molecular markers (Govindaraj *et al.*, 2015). Related to those techniques, the selection of molecular markers is considered more appropriate and effective to avoid any bias due to environmental influence and provide eclectic information about genetic diversity in a more acceptable way (Fu, 2015). Some molecular markers are included and considered particularly promising in helping analyse genetic diversity, such as RAPD, RFLP, and SCAR.

Among those markers, RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) is the most popular marker in many research projects (AlRawashdeh and AlRawashdeh, 2015; Ben-Ari and Lavi, 2012). RAPD constitutes a PCR-based (Polymerase Chain Reaction) technique that involves a primary set with a relatively short size and can be a PCR-based technique that involves a relatively short size and can randomly amplify many DNA segments (Kumari and Thakur, 2014). This technique is equipped with outstanding excellence compared to others, which occupies a universal primary set without undergoing the DNA sequencing phase in its actual implementation. In addition, the RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar and Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari and Thakur, 2014), and is applicable for various laboratory situations (Kumar and Gurusubramanian, 2011). Therefore, RAPD has often been

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used as a genetic marker in much research on the genetic variation of various legumes.

However, analyses of the genetic diversity of *P. lunatus* in Indonesia are still rare. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in North America (Serrano-serrano *et al.*, 2010), Central America (Camacho-Pérez *et al.*, 2018), and South America (Silva *et al.*, 2019). On the one hand, in Indonesia, research about *P. lunatus* was still limited on its potency as an alternative food source (Diniyah *et al.*, 2013; Herry *et al.*, 2014) alongside its essential substances (Diniyah *et al.*, 2015; Praseptiangga *et al.*, 2018; Sukatiningsih *et al.*, 2013; Tejasari, 2016). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti and Fauzi, 2019). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on the RAPD marker.

## 6. Material and Methods

### 6.1. Collection of Samples

*P. lunatus* used in this present study was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. Based on the result of identification in previous research (Purwanti and Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Further, each of the accessions was planted in a polybag in which one polybag was distanced one meter long from the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agents.

**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura
Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

### 6.2. DNA Isolation

DNA isolation was administered based on the CTAB method of Doyle and Doyle (1984), which was modified by Maftuchah and Zainuddin (2010). The used tissue stemmed from the leaf organ of 3 mo (month-old) plants. First, leaves were cut out and were crushed using liquid nitrogen. Then, Natrium bisulfited was weighed for each of 12 samples and dissolved into the buffer. The results of isolated DNA were stored under a temperature of -20 °C.

**Table 2.** List of primers

Primers	Sequence 5'-3'	GC Content (%)
OPA6	GGTCCCTGAC	70
OPA8	GTGACGTAGG	60
OPA10	GTGATCGCAG	60
OPA20	GTTGCGATCC	60
OPC19	GTTGCCAGCC	70
OPD8	GTGTGCCCCA	70
OPD12	CACCGTATCC	60
OPE8	TCACCACGGT	60
OPE15	ACGCACAACC	60
OPE16	GGTGACTGTG	60

### 6.3. PCR-RAPD

Ten primers having 60 to 70 GC content were used in this present study (Table 2). The total volume of PCR reaction used signified 22.5 µL, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and 30 ng DNA template. The condition for PCR reaction was designed under the pre-denaturation temperature of 94 °C (in 5 min), denaturation temperature of 94 °C (in 1 min), primary attachment temperature of 36 °C (in 1 min), extension temperature of 72 °C (in 2 min), the post-extension temperature of 72 °C (in 5 min), and post PCR reaction temperature of 4 °C (in 2 min). For multiplication, the cycle of the PCR reaction was repeated 36 times.

### 6.4. Agarose Gel Electrophoresis

There were three stages of procedure to confirm the result of isolation process and PCR reaction after implementation. The first stage was creating agarose gel with a concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labelled electrophoresis with the electrophoresis buffer of TBE (1×), loading dye (6×) under the condition of 60 V, 400 mA within 45 min. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

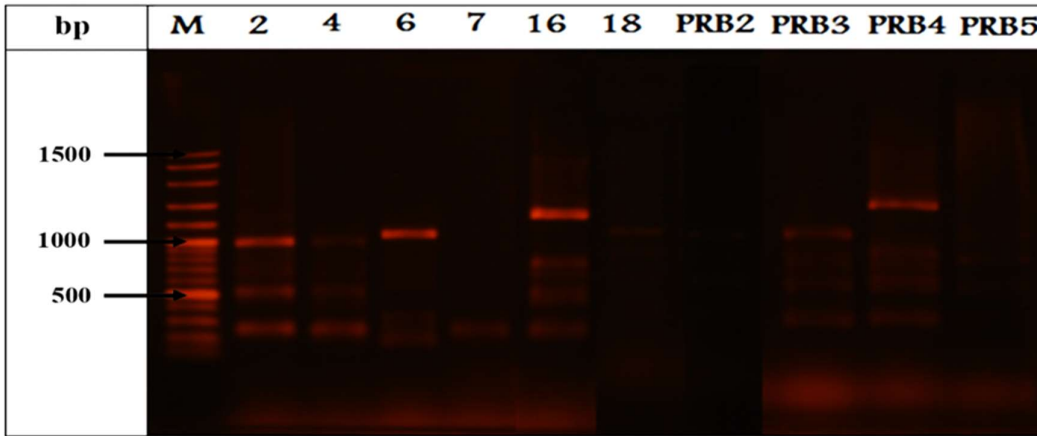
### 6.5. Analysis on the DNA Bands Yielded from RAPD

Data analysis was performed by observing the pattern of visible bands from the electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data (0 and 1), indicating the existence and inexistence of bands typifying specific sizes. Afterward, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and create a phylogenetic tree by creating a phylogenetic tree with Popgen software version 3.1 (Yeh and Boyle, 1997).

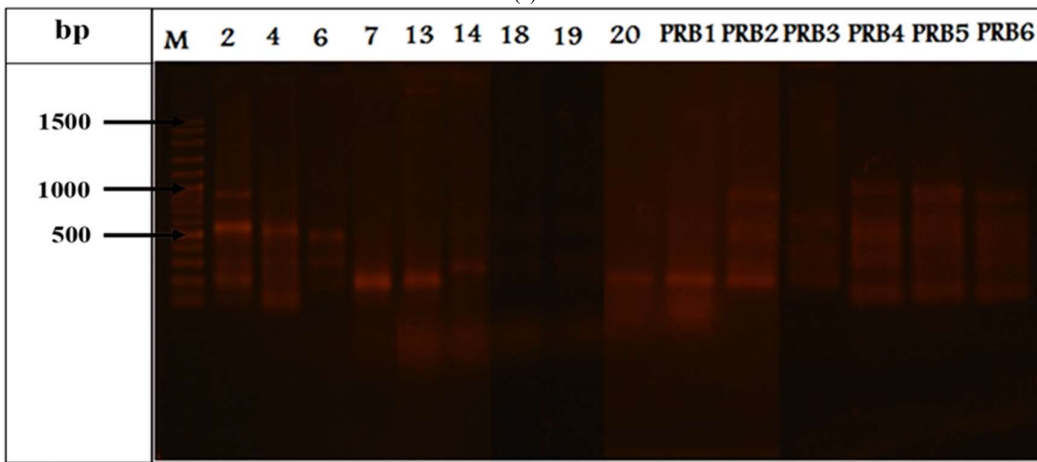
**7. Results and Discussion**

In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, as a result of PCR-RAPD amplification at those genotypes

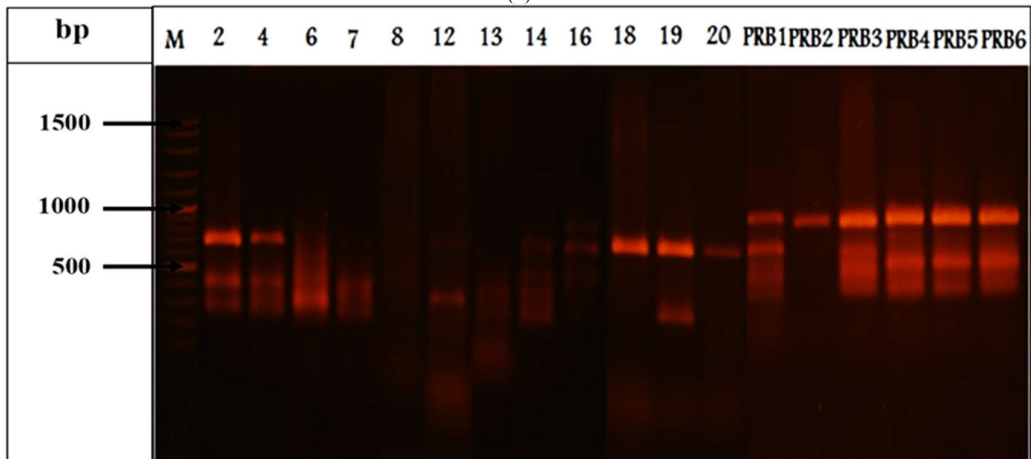
(Figure 1), the bands were assessed based on the binary data, with the description of 1 for the amplified band and 0 for unamplified. The following Table 3 showed the record of the number of loci found.



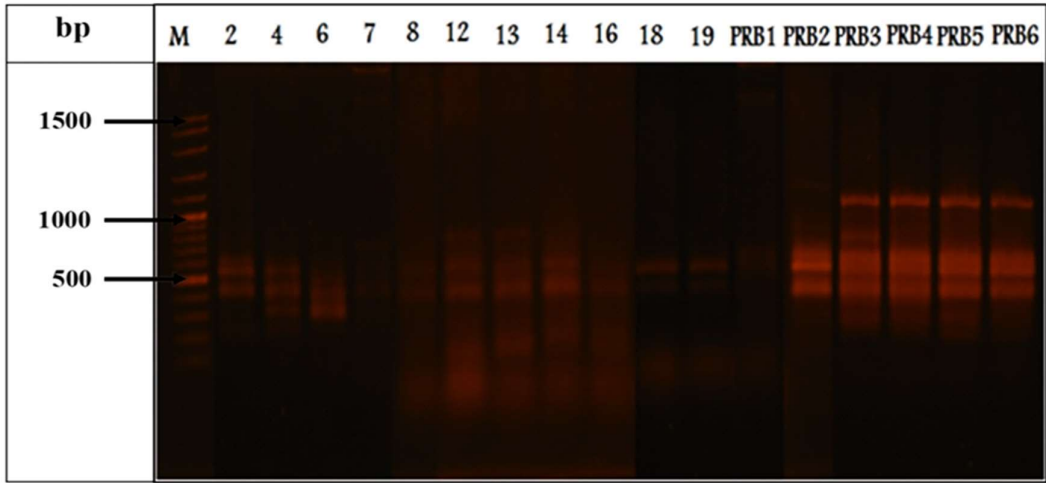
(a)



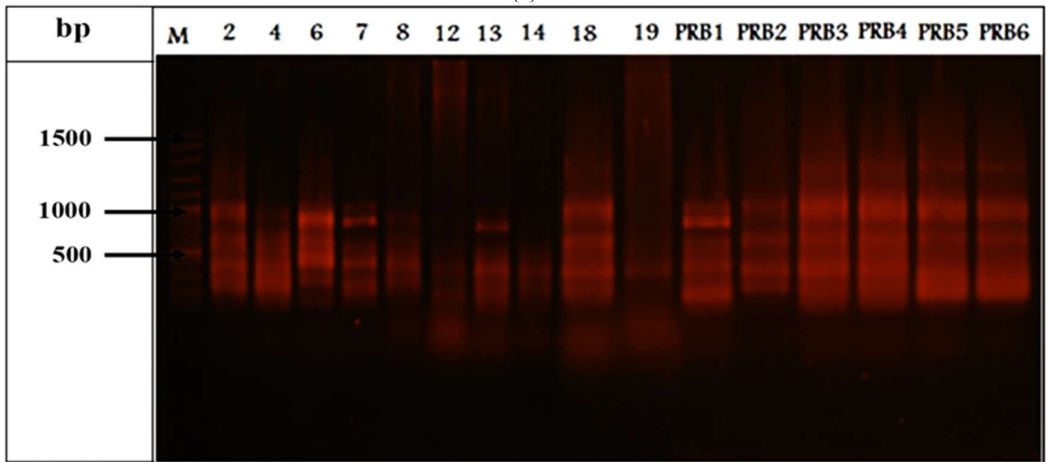
(b)



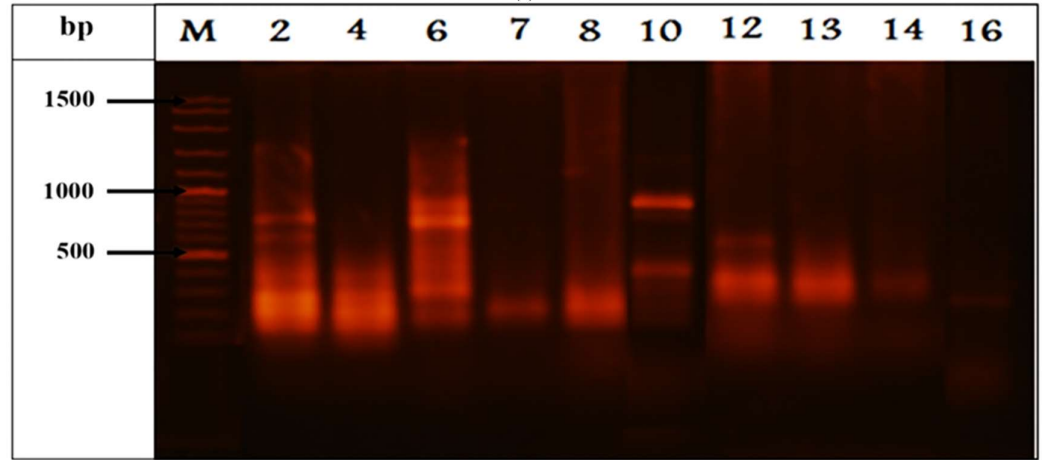
(c)



(d)

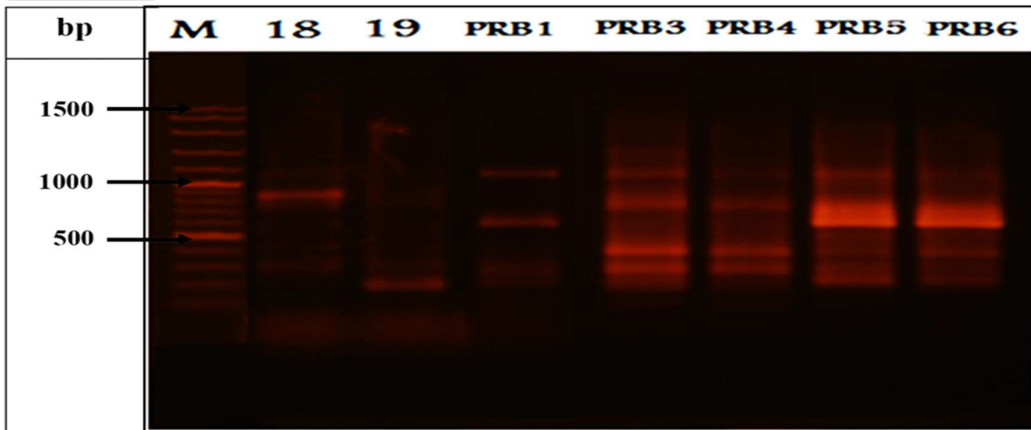
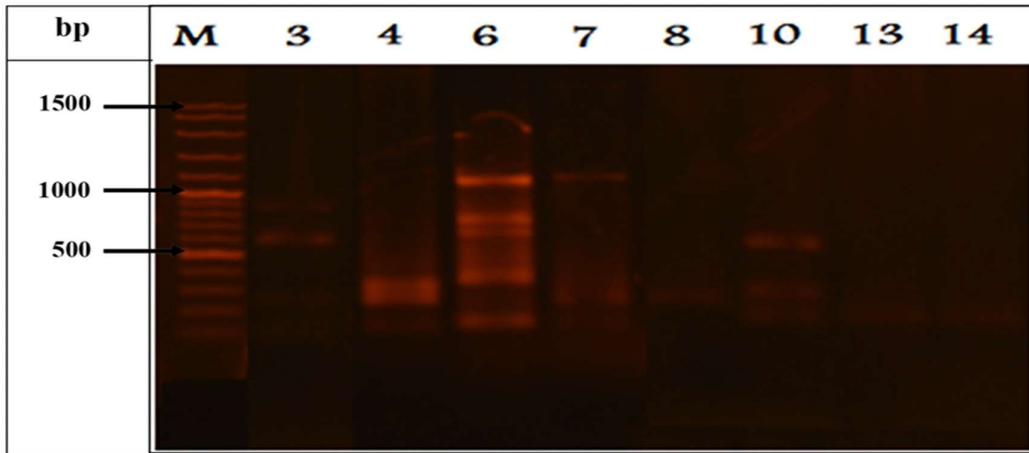


(e)

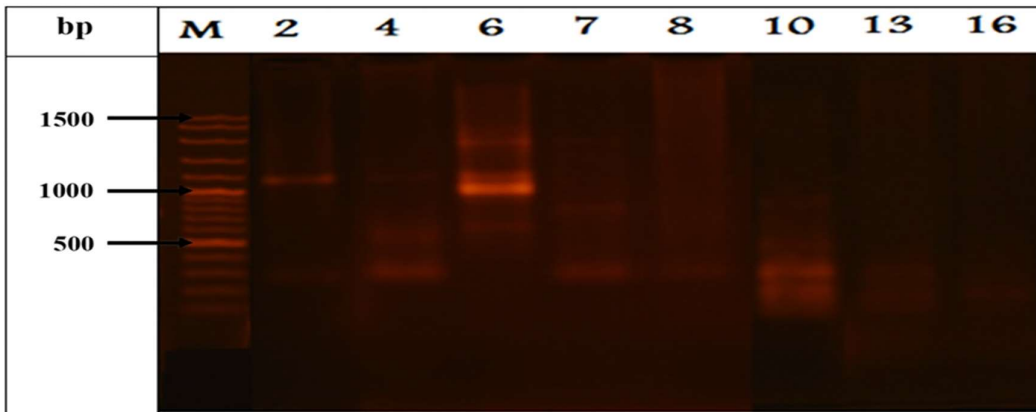


(f)



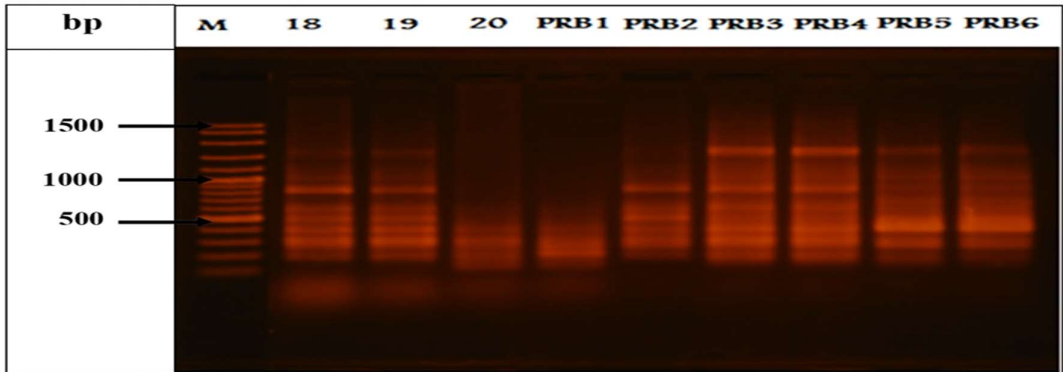
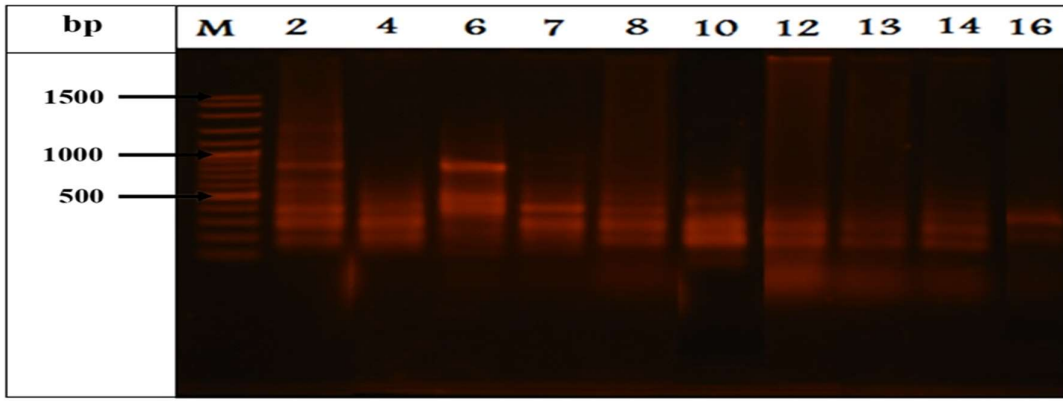


(g)

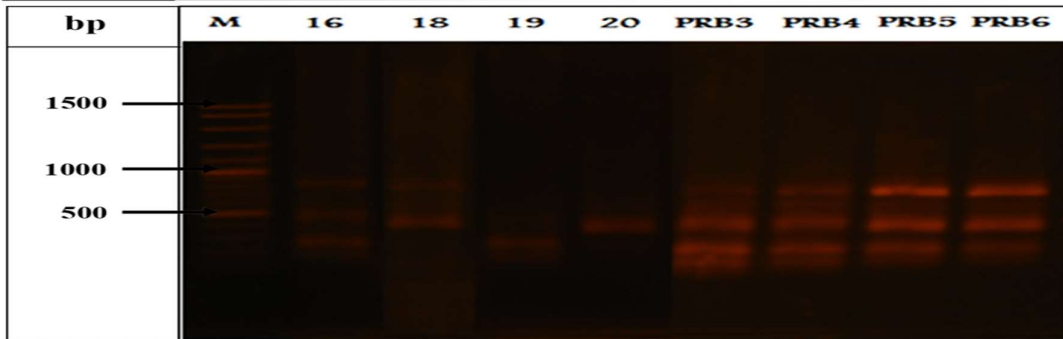
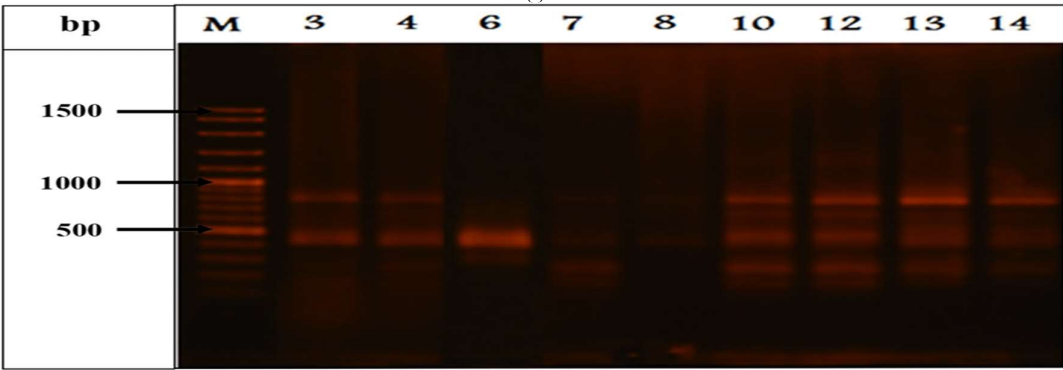


(h)





(i)



(j)

Figure 1. Gel electrophoresis profiles for PCR-RAPD results using (a) OPA6, (b) OPA8, (c) OPA10, (d) OPA20, (e) OPA19, (f) OPD8, (g) OPD12, (h) OPE8, (i) OPE15, (j) OPE16. M is the DNA size ladder

**Table 3.** The record of the number of locus found at 15 genotypes of *P. lunatus* using 10 random primary

Codes	Locus (bp)												
	100	200	300	400	500	600	700	800	900	1000	1100	1200	1300
2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

Description: (1): DNA band was existent, (0): DNA band was non-existent.

After PCR was conducted, DNA fragments of diverse sizes (polymorphic) were produced (Table 4). A total of 68 amplified bands were obtained, out of 67 were polymorphic. The percent polymorphism was 96% to 100%. The total number of amplified bands varied between 5 (OPA10 and

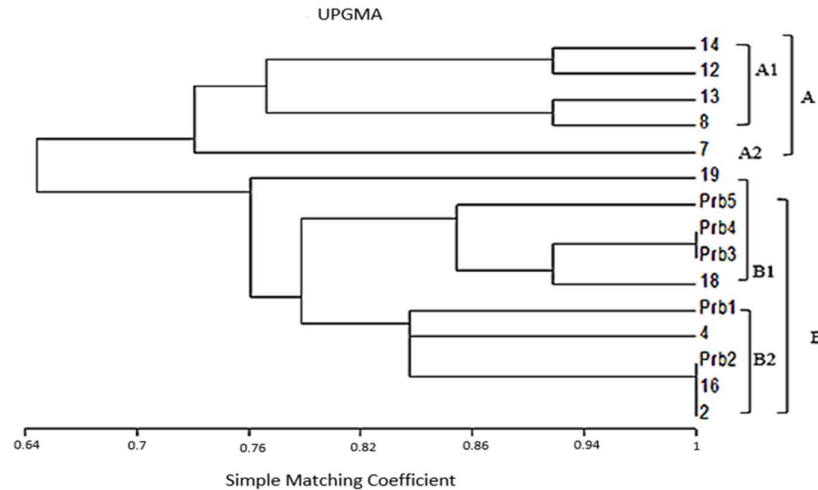
OPA20) to 9 (OPA12), with an average of 6.8 bands per primer. Ten unique bands were detected in several accessions. The size of the unique band ranged from 200 (Mdr6) to 1 300 bp (Prb5).

Table 4. RAPD primers used for diversity analysis of *P. lunatus*.

No.	Markers (100 bp– 2000 bp)	Σ Band	Polymorphic	% Polymorphic	Unique band		
					Total	Locus	Accession
1	OPA 6	8	8	100%	2	700 200	Mdr16 Mdr6
2	OPA 8	7	7	100%	0	-	-
3	OPA 10	5	5	100%	0	-	-
4	OPA 20	5	5	100%	1	300	Mdr4
5	OPC 19	6	5	96%	0	-	-
6	OPD 8	8	8	100%	1	500	Prb1
7	OPD 12	9	9	100%	2	1 200 900	Prb3 Mdr19
8	OPE 8	7	7	100%	2	1 300 500	Prb5 Mdr4
9	OPE 15	7	7	100%	0	-	-
10	OPE 16	6	6	100%	2	700 500	Mdr12 Mdr16
Total		68	67		10		

The cluster analysis upon the 68 RAPD bands was administered. The phylogenetic tree using Neighbor-Joining method was produced, equipped with similarity coefficient that ranged from 64% to 100%, or there was genetic variation with the range of 0% to 36% (Figure 2). In GSC of 0.6, *P. lunatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with GSC of

0.932 (including 8, 12, 13, 14 genotypes), A2 with GSC of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). At GSC of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close.



**Figure 2.** The dendrogram showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* was relatively high (ranging from 96% to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Camacho-Pérez *et al.*, 2018; Chacón-Sánchez and Martínez-Castillo, 2017). The existing high polymorphism had indicated the vast range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes a series of parameters to define genetic diversity on particular species (Singh and Kulathinal, 2013). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of the gene pool. According to the previous study, the 15 accessions of *P. lunatus* included in the current research were originated from five different gene pools, name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti and Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The ecological condition was closely interconnected with the agro-climatic zone in which it was grown. The origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity considered high had brought about an urgent implication in terms of crop improvement attempt, including the breeding procedure for quality improvement (Bhanu, 2017; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not effective as the main food resource (Doria *et al.*, 2012). Consequently, the process of maturation in *P. lunatus* seeds needed proper and acceptable process; thus it could reduce the anti-nutritional substances (Sukatningsih *et al.*, 2013). Further, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factors. In addition, the breeding procedure could be designed that way to yield the generation of *P.*

*lunatus* with highly pest-resistant performance and a shorter period of maturation phase.

Next, concerning the phylogenetic tree formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior. Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width, and pod width compared to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti and Fauzi, 2019).

Despite this fact, cluster analysis also indicated the existence of accessions of which pod lengths were quite different but still grouped in the same cluster. In contrast, when the pod length was not significantly different, cluster analysis classified those accessions in different clusters. This was probably due to DNA related to the RAPD marker used in this current research being unrelated to the character set. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, the use of RAPD was not only affordable but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. However, there were some studies of genetic variation on germplasm of beans that were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye and Ojobo, 2012), Turkey (Ince and Karaca, 2011), and in Vicia Faba, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez and Martínez-Castillo, 2017). In

short, this current research has advocated the credibility of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

## 8. Conclusion

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analysed. The result of RAPD using ten primers resulted in 68 bands in which nine primers possessed 100% level of polymorphism, and one primary was equipped with 96% level of polymorphism. In addition, ten unique bands were detected in eight accessions, i.e. Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6. Based on cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13, and 14, while the latter comprised the 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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# Diversity of *Phaseolus lunatus* L. in East Java, Indonesia based on PCR-RAPD technique

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## Abstract

*Phaseolus lunatus* L. is one of the legume plants found in some parts of Indonesia and has potential as alternative food rich in protein. This current research aimed at analysing genetic accessions of *P. lunatus* distributed in some areas in East Java, Indonesia, based on the RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) marker. 15 accessions originated from four locations were analysed. Ten primers were used and produced 68 bands out of 67 were polymorphic. The percent polymorphism was 96% to 100%. Ten unique bands were detected in eight accessions (Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6). Using the Neighbor-Joining method, a phylogenetic tree was yielded by a similarity coefficient of 64% to 100%. On the genetic similarity coefficient (GSC) of 0.6, there were two clusters: the first and second major clusters (Cluster A and B). The former contained the accessions 7, 8, 13, and 14, while the latter comprised 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5. In conclusion, based on phylogenetic trees formed, *P. lunatus* from the same region cluster in the same cluster.

**Keywords:** Genetic diversity; Lima bean, Phylogeny, Polymerase Chain Reaction, Random Amplified Polymorphic Deoxyribonucleic acid

## 1. Introduction

*Phaseolus lunatus* L. is categorized as a legume plant with great potential to become nutritious food. In Indonesia, *P. lunatus* can be found in some islands, such as Java and Madura (Purwanti and Fauzi, 2019). The distribution of *P. lunatus* in this country implies the probability of various accessions existing in Indonesia. The diversity of *P. lunatus* accessions and relative ease offered to cultivate the plant bring about probability for Indonesian societies as well as government to make use of it as an alternatively functional food source (Diniyah *et al.*, 2013; Diniyah *et al.*, 2015; Herry *et al.*, 2013; Nafi *et al.*, 2015). Nowadays, Indonesia is dealing with the severe issue of protein shortage in some areas (Diana *et al.*, 2017; Ickowitz *et al.*, 2016; Madanijah *et al.*, 2016), so maximizing consumer consumption *P. lunatus* will be an effective solution.

With respect to elevating *P. lunatus* as one of food sources, identification on genetic diversity typifying the accessions with high protein content needs actualization. The obtainability of information regarding genetic diversity of intra- and inter-species is the most essential foundation to run all the programs of food source enhancement (Bhanu, 2017). Also, information that pinpoints natural variability and difference that lies on the plant itself is used as the primary capital to design a

betterment scheme for the species since the beginning of systematic plant breeding (Bhanu, 2017). Furthermore, such information can be used to reach a phase of sustainable crop production (Fu, 2015). Moreover, the research underpinning genetic diversity in a particular plant also leads to conservation (Carvalho *et al.*, 2019). For that reason, to support the attempt, a series of assessments on genetic diversity have been routinely administered using numerous techniques such as morphological identification, biochemical characterization, and analysis of molecular markers (Govindaraj *et al.*, 2015). Related to those techniques, the selection of molecular markers is considered more appropriate and effective to avoid any bias due to environmental influence and provide eclectic information about genetic diversity in a more acceptable way (Fu, 2015). Some molecular markers are included and considered particularly promising in helping analyse genetic diversity, such as RAPD, RFLP, and SCAR.

Among those markers, RAPD (Random Amplified Polymorphic Deoxyribonucleic acid) is the most popular marker in many research projects (AIRawashdeh and AIRawashdeh, 2015; Ben-Ari and Lavi, 2012). RAPD constitutes a PCR-based (Polymerase Chain Reaction) technique that involves a primary set with a relatively short size and can be a PCR-based technique that involves a relatively short size and can randomly amplify many DNA segments (Kumari and Thakur, 2014). This

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technique is equipped with outstanding excellence compared to others, which occupies a universal primary set without undergoing the DNA sequencing phase in its actual implementation. In addition, the RAPD marker is effective to demonstrate reasonable speed and is deemed more efficient (Kumar and Gurusubramanian, 2011), as it can be used for limited DNA samples, is not costly (Kumari and Thakur, 2014), and is applicable for various laboratory situations (Kumar and Gurusubramanian, 2011). Therefore, RAPD has often been used as a genetic marker in much research on the genetic variation of various legumes.

However, analyses of the genetic diversity of *P. lunatus* in Indonesia are still rare. In fact, in some parts of the world, such kinds of analyses are intensively published, such as in North America (Serrano-serrano *et al.*, 2010), Central America (Camacho-Pérez *et al.*, 2018), and South America (Silva *et al.*, 2019). On the one hand, in Indonesia, research about *P. lunatus* was still limited on its potency as an alternative food source (Diniyah *et al.*, 2013; Herry *et al.*, 2014) alongside its essential substances (Diniyah *et al.*, 2015; Praseptianga *et al.*, 2018; Sukatiningsih *et al.*, 2013; Tejasari, 2016). In addition, researches that study the diversity of *P. lunatus* are still restricted to its morphological characteristics (Purwanti and Fauzi, 2019). Therefore, this research is focused on the genetic diversity of *P. lunatus* based on the RAPD marker.

## 2. Material and Methods

### 2.1. Collection of Samples

*P. lunatus* used in this present study was originated from seeds collected from some areas in East Java, Indonesia, such as Madura, Tulungagung, Malang, and Probolinggo. Based on the result of identification in previous research (Purwanti and Fauzi, 2019), the collection of *P. lunatus* consisted of 15 accessions. All those fifteen are listed in the following Table 1. Further, each of the accessions was planted in a polybag in which one polybag was distanced one meter long from the next one. In addition, the plantation was done without any extraneous additions of neither fertilizer nor other kinds of growth agents.

**Table 1.** List of accessions to analyze

Accession Codes	Origins
2	Madura
4	Madura
7	Madura
8	Madura
12	Madura
13	Madura
14	Madura, Tulungagung
16	Madura
18	Madura
19	Madura
Prb1	Probolinggo
Prb2	Probolinggo
Prb3	Probolinggo
Prb4	Probolinggo, Malang
Prb5	Probolinggo, Madura

### 2.2. DNA Isolation

DNA isolation was administered based on the CTAB method of Doyle and Doyle (1984), which was modified by Maftuchah and Zainuddin (2010). The used tissue stemmed from the leaf organ of 3 mo (month-old) plants. First, leaves were cut out and were crushed using liquid nitrogen. Then, Natrium bisulfited was weighed for each of 12 samples and dissolved into the buffer. The results of isolated DNA were stored under a temperature of -20 °C.

**Table 2.** List of primers

Primers	Sequence 5'-3'	GC Content (%)
OPA6	GGTCCCTGAC	70
OPA8	GTGACGTAGG	60
OPA10	GTGATCGCAG	60
OPA20	GTTGCGATCC	60
OPC19	GTTGCCAGCC	70
OPD8	GTGTGCCCCA	70
OPD12	CACCGTATCC	60
OPE8	TCACCACGGT	60
OPE15	ACGCACAACC	60
OPE16	GGTGACTGTG	60

### 2.3. PCR-RAPD

Ten primers having 60 to 70 GC content were used in this present study (Table 2). The total volume of PCR reaction used signified 22.5 µL, containing the mixture of liquid DNA of *taq* polymerase and 10-fold buffer of *taq* polymerase (100 mM Tris-Cl, pH 8.3; 500 mM KCl; 15 mM MgCl<sub>2</sub>; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH<sub>2</sub>O; and 30 ng DNA template. The condition for PCR reaction was designed under the pre-denaturation temperature of 94 °C (in 5 min), denaturation temperature of 94 °C (in 1 min), primary attachment temperature of 36 °C (in 1 min), extension temperature of 72 °C (in 2 min), the post-extension temperature of 72 °C (in 5 min), and post PCR reaction temperature of 4 °C (in 2 min). For multiplication, the cycle of the PCR reaction was repeated 36 times.

### 2.4. Agarose Gel Electrophoresis

There were three stages of procedure to confirm the result of isolation process and PCR reaction after implementation. The first stage was creating agarose gel with a concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labelled electrophoresis with the electrophoresis buffer of TBE (1×), loading dye (6×) under the condition of 60 V, 400 mA within 45 min. At last, the stage was the coloration using 10%-concentrated ethidium-bromide and the documentation of the DNA using UV-Trans illuminator.

### 2.5. Analysis on the DNA Bands Yielded from RAPD

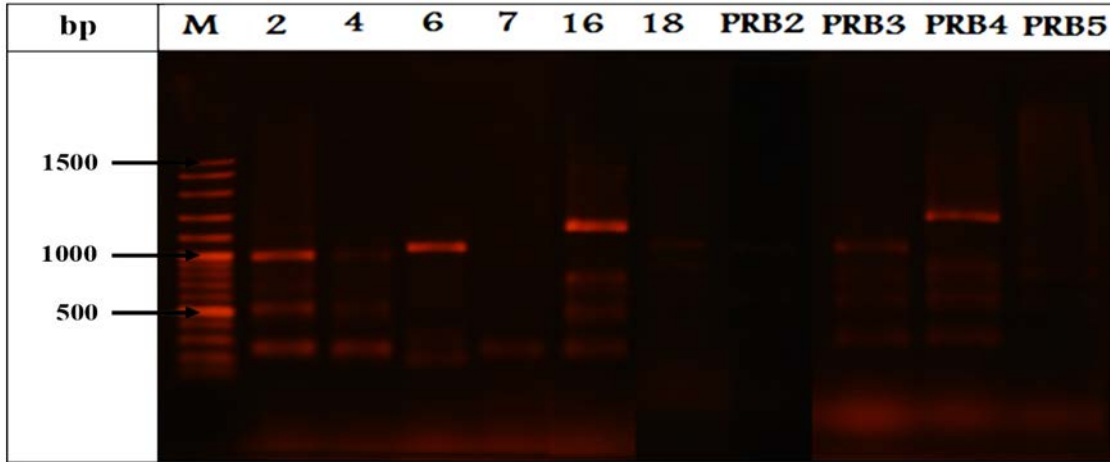
Data analysis was performed by observing the pattern of visible bands from the electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data (0 and 1), indicating the existence and inexistence of bands typifying specific sizes. Afterward, the existing bands were observed to identify the percentage of polymorphic and monomorphic bands and create a phylogenetic tree by creating a phylogenetic tree with Popgen software version 3.1 (Yeh and Boyle, 1997).



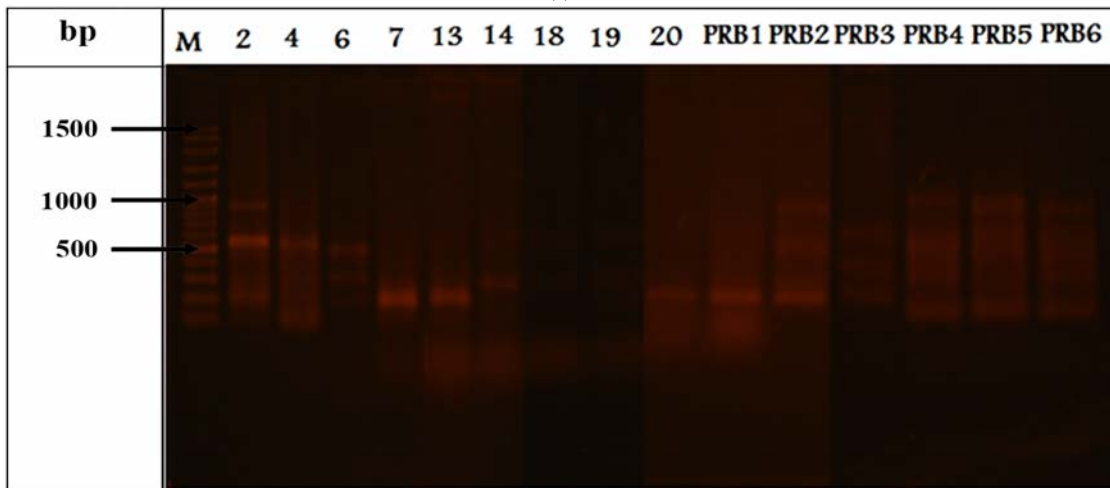
**3. Results and Discussion**

In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, as a result of PCR-RAPD amplification at those

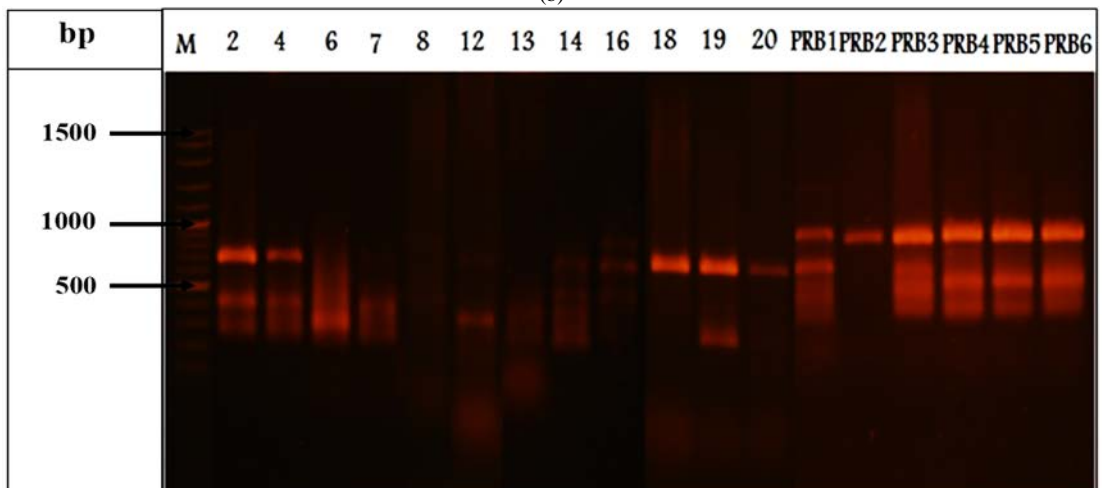
genotypes (Figure 1), the bands were assessed based on the binary data, with the description of 1 for the amplified band and 0 for unamplified. The following Table 3 showed the record of the number of loci found.



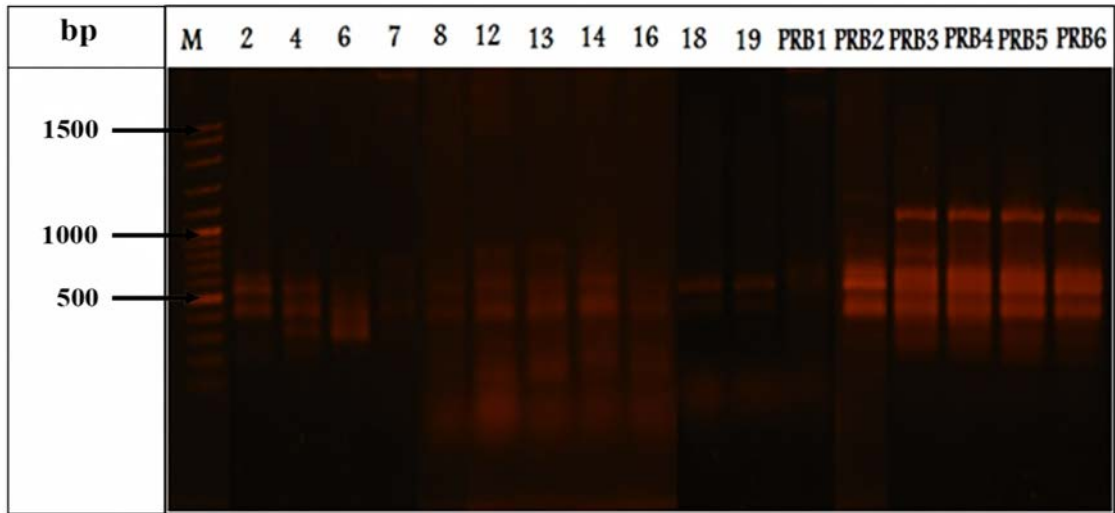
(a)



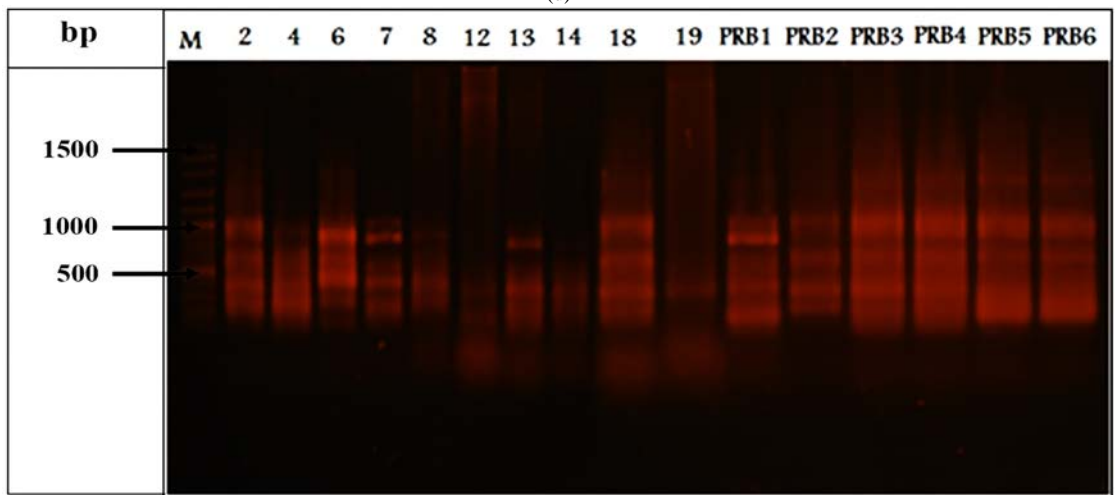
(b)



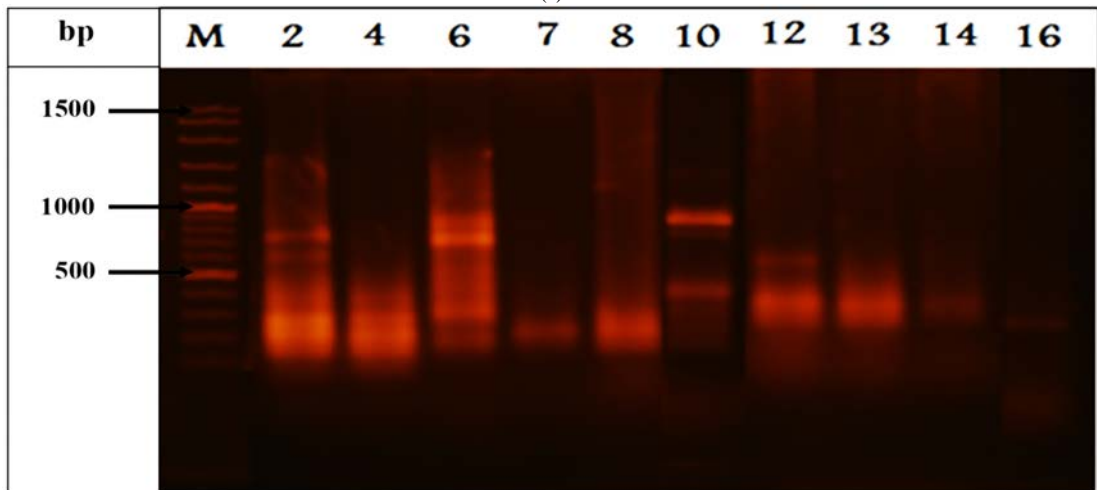
(c)



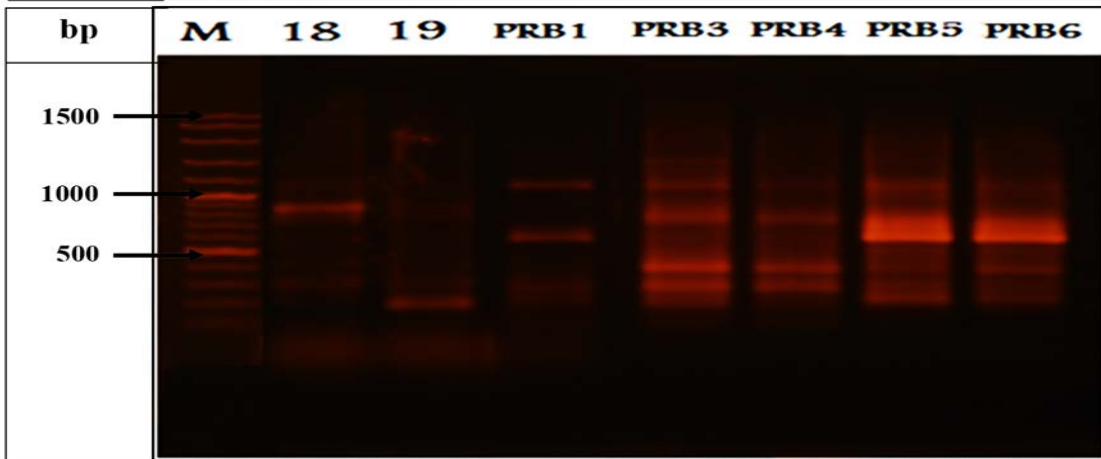
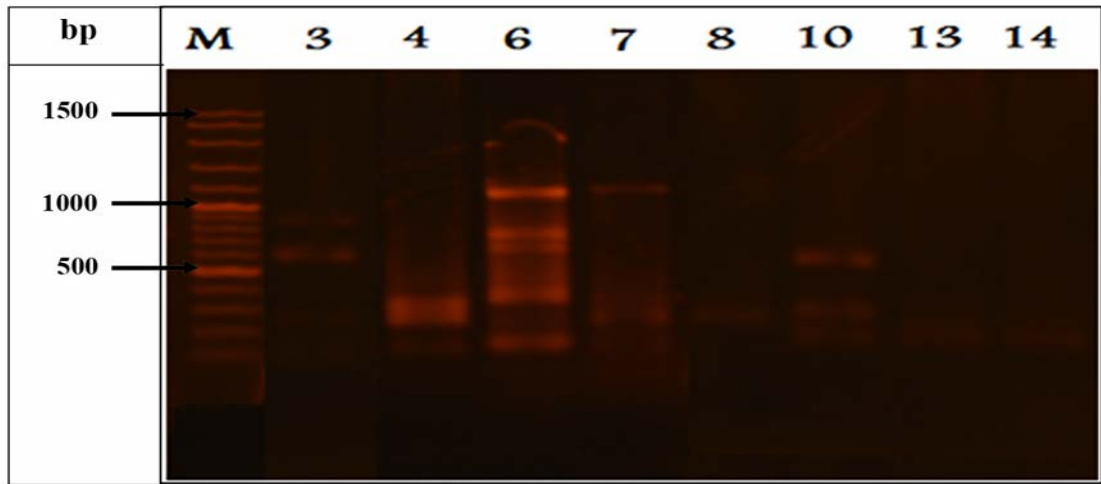
(d)



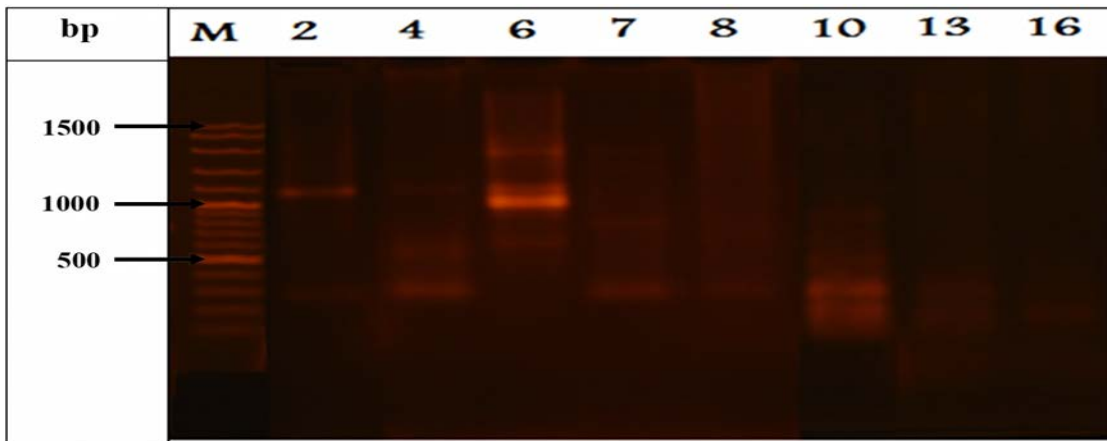
(e)



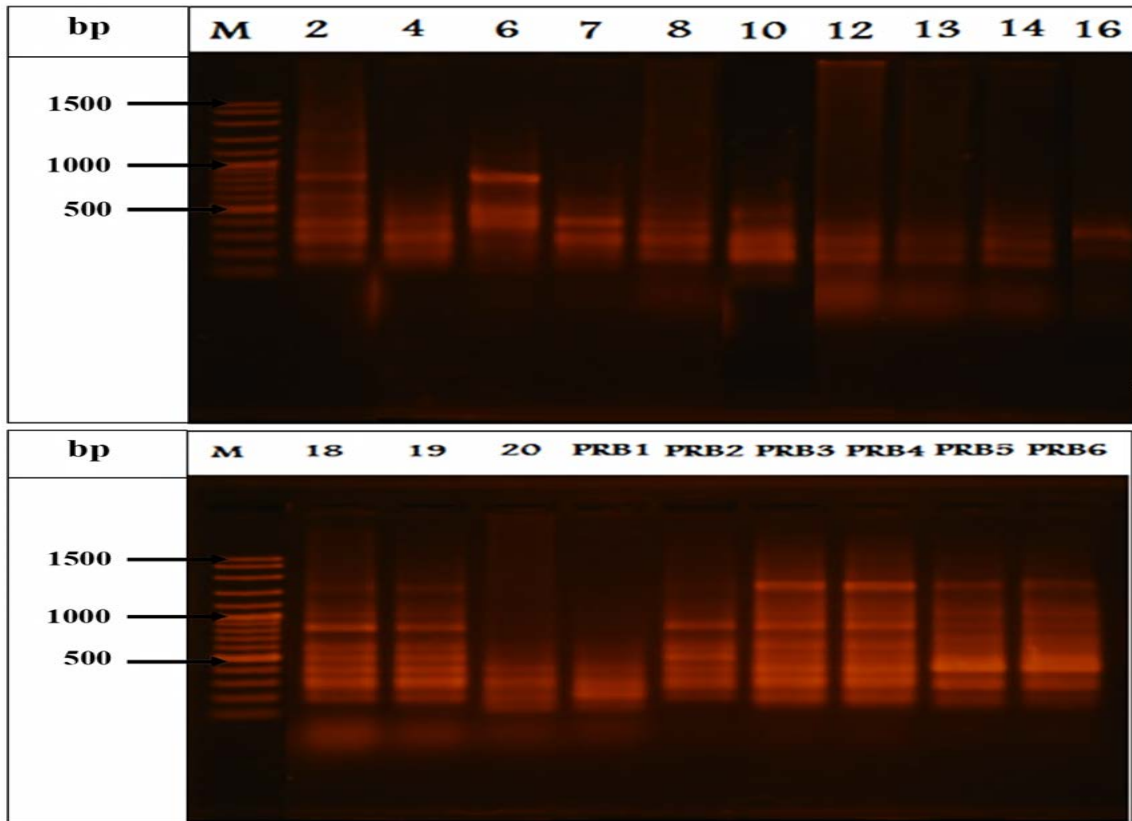
(f)



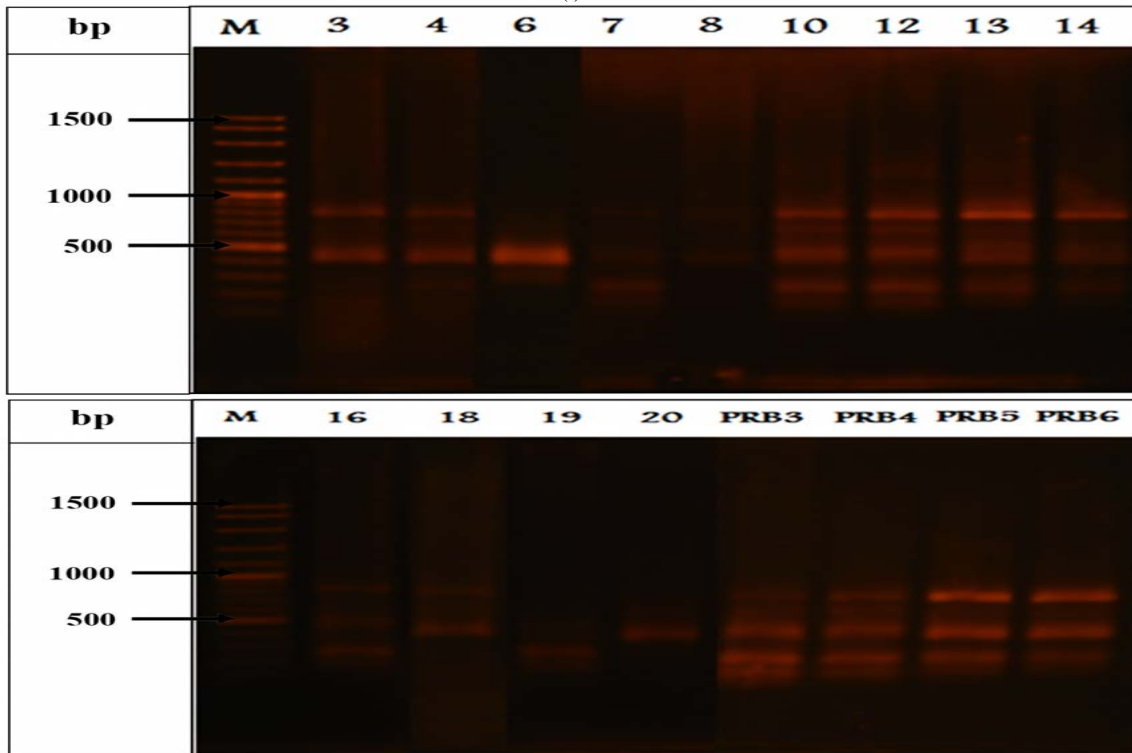
(g)



(h)



(i)



(j)

**Figure 1.** Gel electrophoresis profiles for PCR-RAPD results using (a) OPA6, (b) OPA8, (c) OPA10, (d) OPA20, (e) OPA19, (f) OPD8, (g) OPD12, (h) OPE8, (i) OPE15, (j) OPE16. M is the DNA size ladder

**Table 3.** The record of the number of locus found at 15 genotypes of *P. lumatus* using 10 random primary

Codes	Locus (bp)												
	100	200	300	400	500	600	700	800	900	1000	1100	1200	1300
2	0	1	1	1	1	1	1	1	1	1	1	0	0
4	1	1	1	1	1	1	1	1	0	1	1	0	0
7	1	1	1	1	0	0	1	1	0	1	1	0	0
8	0	1	1	1	1	0	0	1	0	1	0	0	0
12	1	1	1	1	1	1	1	1	0	0	0	0	0
13	1	1	1	1	1	0	0	1	0	1	0	0	0
14	1	1	1	1	1	0	1	1	0	0	0	0	0
16	0	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	0	1	1	0
19	0	1	1	1	1	1	1	1	0	0	1	1	0
Prb1	1	1	1	1	1	1	1	0	1	1	1	0	0
Prb2	0	1	1	1	1	1	1	1	1	1	1	0	0
Prb3	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb4	1	1	1	1	1	1	1	1	1	1	1	1	0
Prb5	1	1	1	1	1	1	1	1	1	0	1	1	1

Description: (1): DNA band was existent, (0): DNA band was non-existent.

After PCR was conducted, DNA fragments of diverse sizes (polymorphic) were produced (Table 4). A total of 68 amplified bands were obtained, out of 67 were polymorphic. The percent polymorphism was 96% to 100%. The total number of amplified bands varied

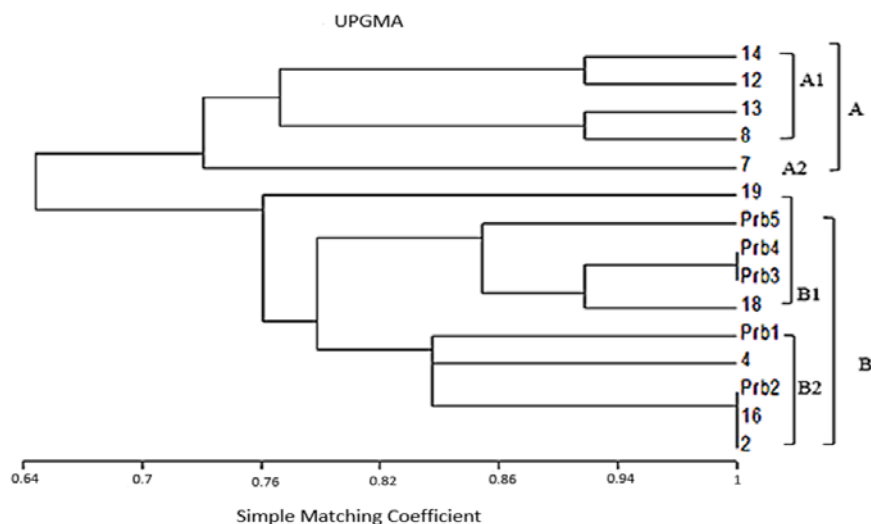
between 5 (OPA10 and OPA20) to 9 (OPA12), with an average of 6.8 bands per primer. Ten unique bands were detected in several accessions. The size of the unique band ranged from 200 (Mdr6) to 1 300 bp (Prb5).

**Table 4.** RAPD primers used for diversity analysis of *P. lumatus*.

No.	Markers (100 bp–2000 bp)	Σ Band	Polymorphic	% Polymorphic	Unique band		
					Total	Locus	Accession
1	OPA 6	8	8	100%	2	700 200	Mdr16 Mdr6
2	OPA 8	7	7	100%	0	-	-
3	OPA 10	5	5	100%	0	-	-
4	OPA 20	5	5	100%	1	300	Mdr4
5	OPC 19	6	5	96%	0	-	-
6	OPD 8	8	8	100%	1	500	Prb1
7	OPD 12	9	9	100%	2	1 200 900	Prb3 Mdr19
8	OPE 8	7	7	100%	2	1 300 500	Prb5 Mdr4
9	OPE 15	7	7	100%	0	-	-
10	OPE 16	6	6	100%	2	700 500	Mdr12 Mdr16
Total		68	67		10		

The cluster analysis upon the 68 RAPD bands was administered. The phylogenetic tree using Neighbor-Joining method was produced, equipped with similarity coefficient that ranged from 64% to 100%, or there was genetic variation with the range of 0% to 36% (Figure 2). In GSC of 0.6, *P. lumatus* accessions were formed into two main clusters, i.e. Cluster A (comprising 7, 8, 13, 14 genotypes) and Cluster B (comprising 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, Prb5 genotypes). Cluster A contained several sub-clusters, which were A1 with GSC of 0.932

(including 8, 12, 13, 14 genotypes), A2 with GSC of 0.73 (including genotype 7). Meanwhile, Cluster B comprised several sub-clusters, too, with B1 (including 19, Prb5, Prb4, Prb3, 18 genotypes) and B2 (including Prb1, 4, Prb2, 16, 2 genotypes). At GSC of 1.0, there were 2, 4, 16, Prb2, Prb3, Prb4 genotypes. The coefficient was considered higher if it approached the point of 1, indicating that the genetic similarity amongst the genotypes was significantly close.



**Figure 2.** The dendrogram showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

*P. lunatus* under analysis through this current research was planted using conventional method in some parts of Indonesia of which environmental conditions were of diversity. The result of analysis indicated that the polymorphic level of *P. lunatus* was relatively high (ranging from 96% to 100%). This kind of finding was in line with previous studies that discussed genetic diversity of *P. lunatus* in some areas of Mesoamerica (Camacho-Pérez *et al.*, 2018; Chacón-Sánchez and Martínez-Castillo, 2017). The existing high polymorphism had indicated the vast range of genetic diversity from the accessions under analysis.

In nature, polymorphism constitutes a series of parameters to define genetic diversity on particular species (Singh and Kulathinal, 2013). The level of polymorphism in intra-species depended on the level of divergence between the genotypes. Regarding the information, *P. lunatus* used in this research was originated from different types of the gene pool. According to the previous study, the 15 accessions of *P. lunatus* included in the current research were originated from five different gene pools, name Sieva-Big, Potato-Sieva, Big lima, Sieva, and Potato-Sieva (Purwanti and Fauzi, 2019). Genetic diversity, moreover, was also a result of ecological situations that differed (Huang *et al.*, 2016). The ecological condition was closely interconnected with the agro-climatic zone in which it was grown. The origins where the samples were taken had different agro-climatic zone (Syuaib, 2016).

The genetic diversity considered high had brought about an urgent implication in terms of crop improvement attempt, including the breeding procedure for quality improvement (Bhanu, 2017; Fu, 2015). As previously reported, *P. lunatus* contained anti-nutritional components that became a confounding factor why its seeds were not effective as the main food resource (Doria *et al.*, 2012). Consequently, the process of maturation in *P. lunatus* seeds needed proper and acceptable process; thus it could reduce the anti-nutritional substances (Sukatiningsih *et al.*, 2013). Further, it was quite probable that the generation of *P. lunatus* would remain low anti-nutritional or without anti-nutritional factors. In addition, the breeding procedure could be designed that way to yield the generation of

*P. lunatus* with highly pest-resistant performance and a shorter period of maturation phase.

Next, concerning the phylogenetic tree formed, the population tended to be clustered based on the geographical origins of those accessions. In fact, Madura accessions were more dominant within Cluster A, while in Cluster B, Probolinggo clusters were superior. Furthermore, there was also shown a trend of connection between similarity of morphological characteristics amongst accessions grouped in the same cluster. Previous studies have reported that most of Probolinggo accessions possessed better characteristics of seed weight, seed length, leaf length, leaf width, and pod width compared to other accessions. Accession 4 constituted the one of which morphological character was close in characteristics to Probolinggo accessions (Purwanti and Fauzi, 2019).

Despite this fact, cluster analysis also indicated the existence of accessions of which pod lengths were quite different but still grouped in the same cluster. In contrast, when the pod length was not significantly different, cluster analysis classified those accessions in different clusters. This was probably due to DNA related to the RAPD marker used in this current research being unrelated to the character set. For that reason, a further study that highlights types of gen is needed to encode those characters.

In accordance with this research, the use of RAPD was not only affordable but also not complex. Also, it could be used to cluster a collection of accessions that could be connected to their agronomic characteristics. Further, reproducibility and sustainability of RAPD marker in the study of genetic variation were still contested in some previous researches. However, there were some studies of genetic variation on germplasm of beans that were successful by utilizing RAPD, such as some researches about *P. vulgaris* in India (Bukhari *et al.*, 2015), South Africa (Adesoye and Ojobo, 2012), Turkey (Ince and Karaca, 2011), and in Vicia Faba, Palestine (Basheer-Salimia *et al.*, 2013). In fact, the result of RAPD shown in this current research shared information which was in line with other generic variation researches by the use of other types of marker, such as ISSR (Camacho-Pérez *et al.*, 2018) and SNP (Chacón-Sánchez and Martínez-Castillo,

2017). In short, this current research has advocated the credibility of RAPD in the study of genetic variation in beans, such as in *P. lunatus*.

#### 4. Conclusion

In this research, the genetic variations of 15 accessions of *P. lunatus* originated from Malang, Probolinggo, Tulungagung, and Madura were analysed. The result of RAPD using ten primers resulted in 68 bands in which nine primers possessed 100% level of polymorphism, and one primary was equipped with 96% level of polymorphism. In addition, ten unique bands were detected in eight accessions, i.e. Prb1, Prb2, Prb5, Mdr12, Mdr16, Mdr19, Mdr4, and Mdr6. Based on cluster analysis, there were two major clusters, Cluster A and B. The former contained the accessions of 7, 8, 13, and 14, while the latter comprised the 2, 4, 16, 18, Prb1, Prb2, Prb3, Prb4, and Prb5.

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