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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 (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

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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; 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. 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 ºC.

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-CI, pH 8.3; 500 mM KCl; 15 mM $MgCI_2$; 0.01% gelatin); *dNTP'S mix* (dGTP, dATP, dTTP and dCTP) (Roche); dH_20; 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

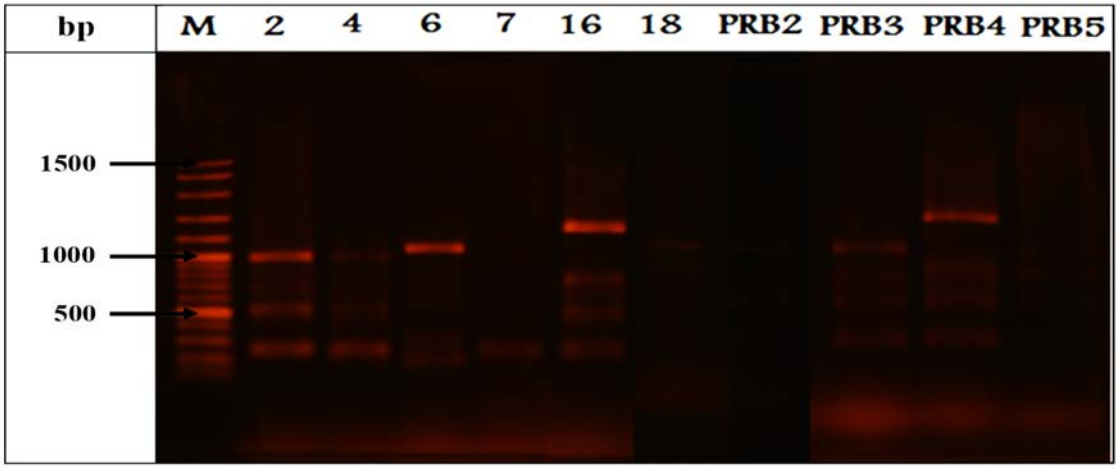
There were three stages of procedure to confirm the result of isolation process and PCR reaction after implementation. The first stage was creating agarose gel with a concentration of 0.8% (for isolation result) and 1% (for PCR result) as a medium of running DNA. Next, the stage was labelled electrophoresis 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

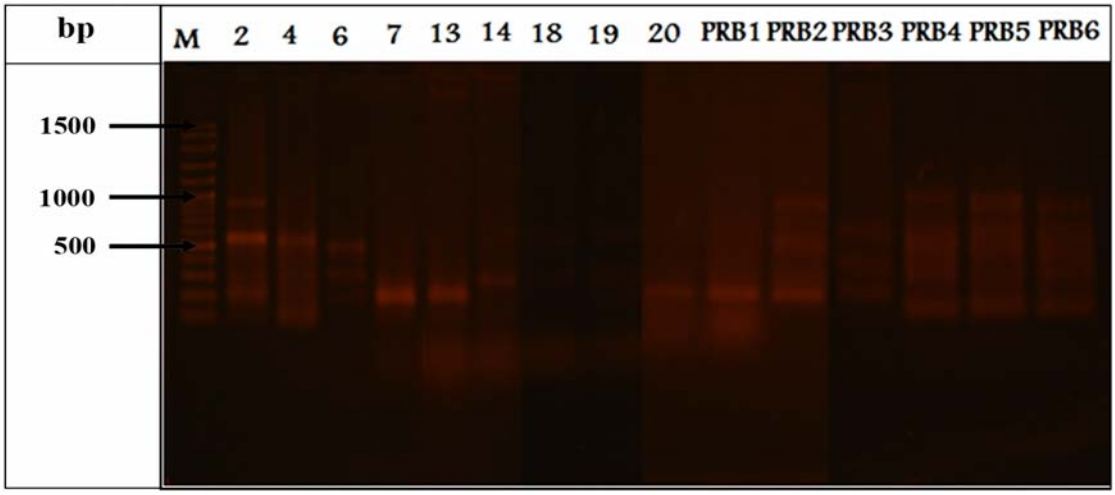
Data analysis was performed by observing the pattern of visible bands from the electrophoresis process in each primary locus. In addition, DNA bands were converted into binary data (0 and 1), indicating the existence and inexistence of bands typifying specific sizes. Afterward, the existing bands were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

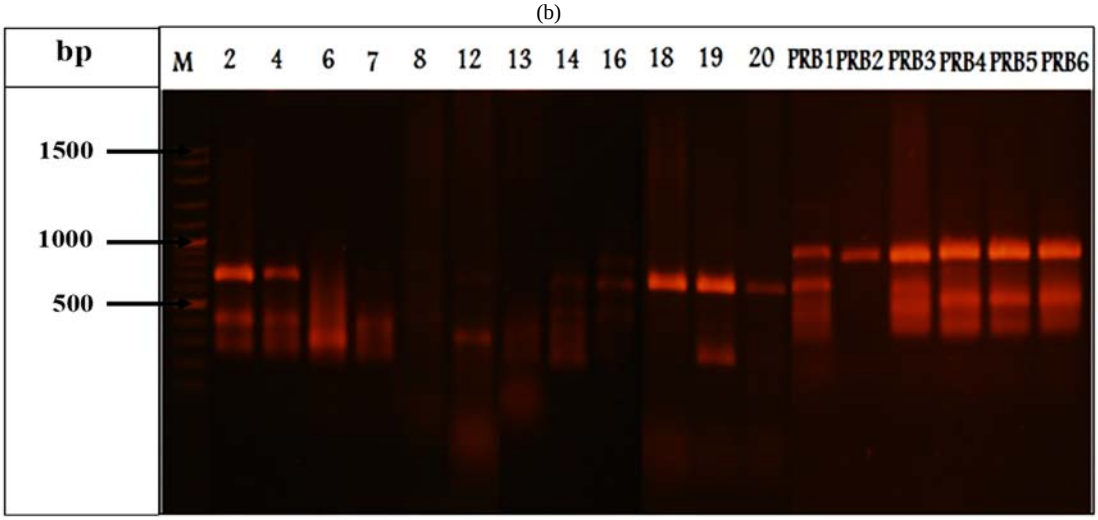
In this current research, ten primers were occupied for DNA amplification at 15 genotypes of *P. lunatus* L. Further, as a result of PCR–RAPD amplification at those genotypes (Figure 1), the bands were assessed based on the binary data, with the description of 1 for the amplified band and 0 for unamplified.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



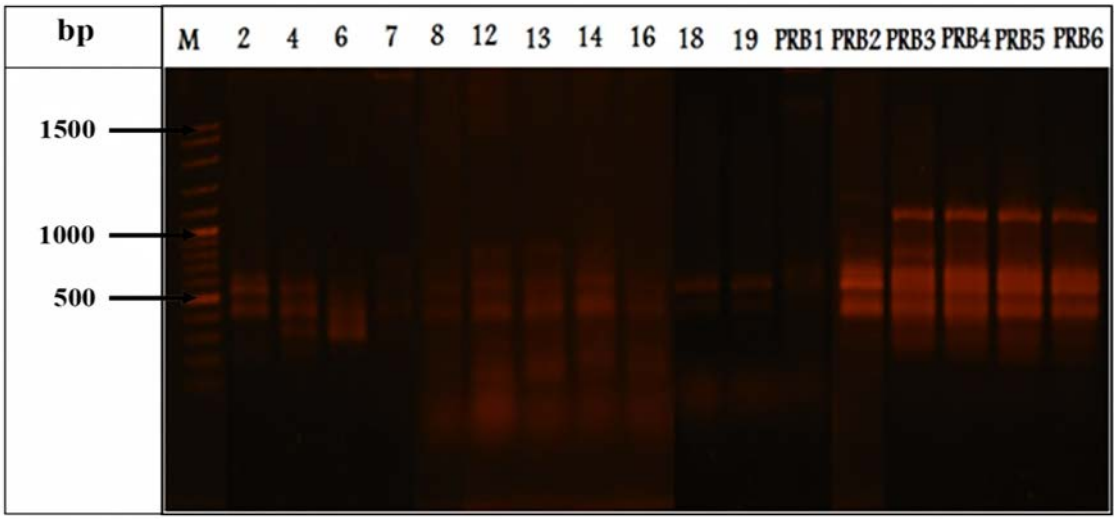
(a)



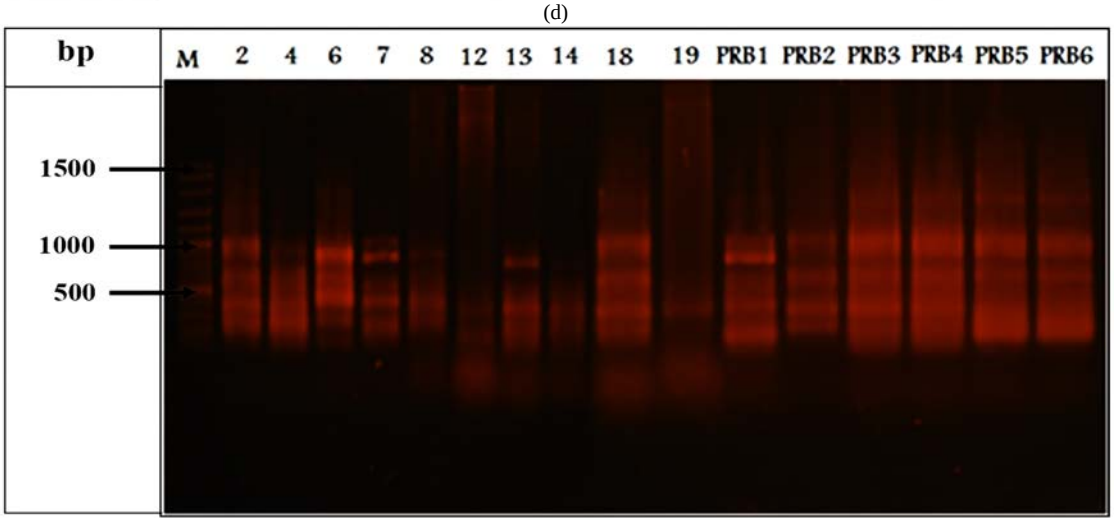
(b)



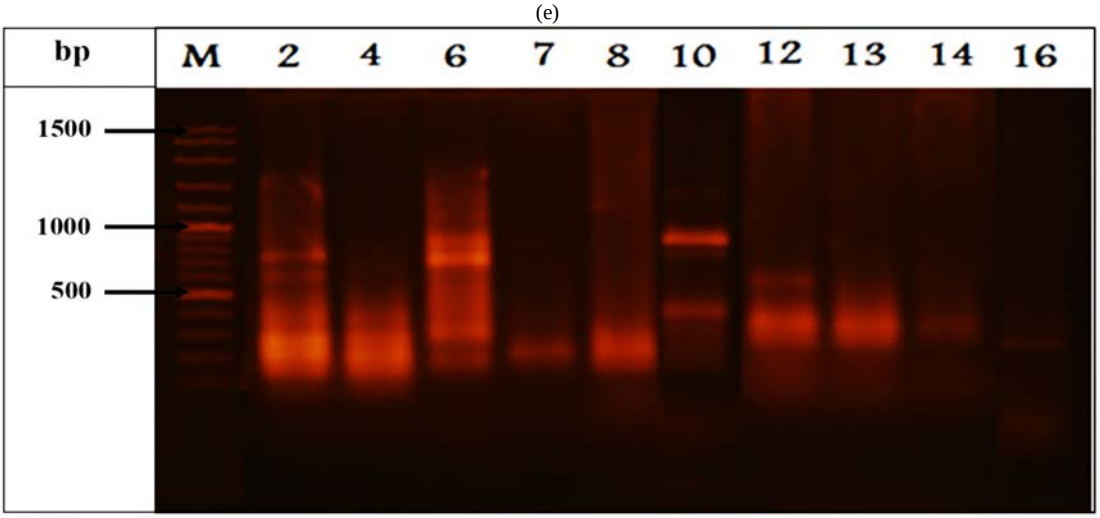
(c)



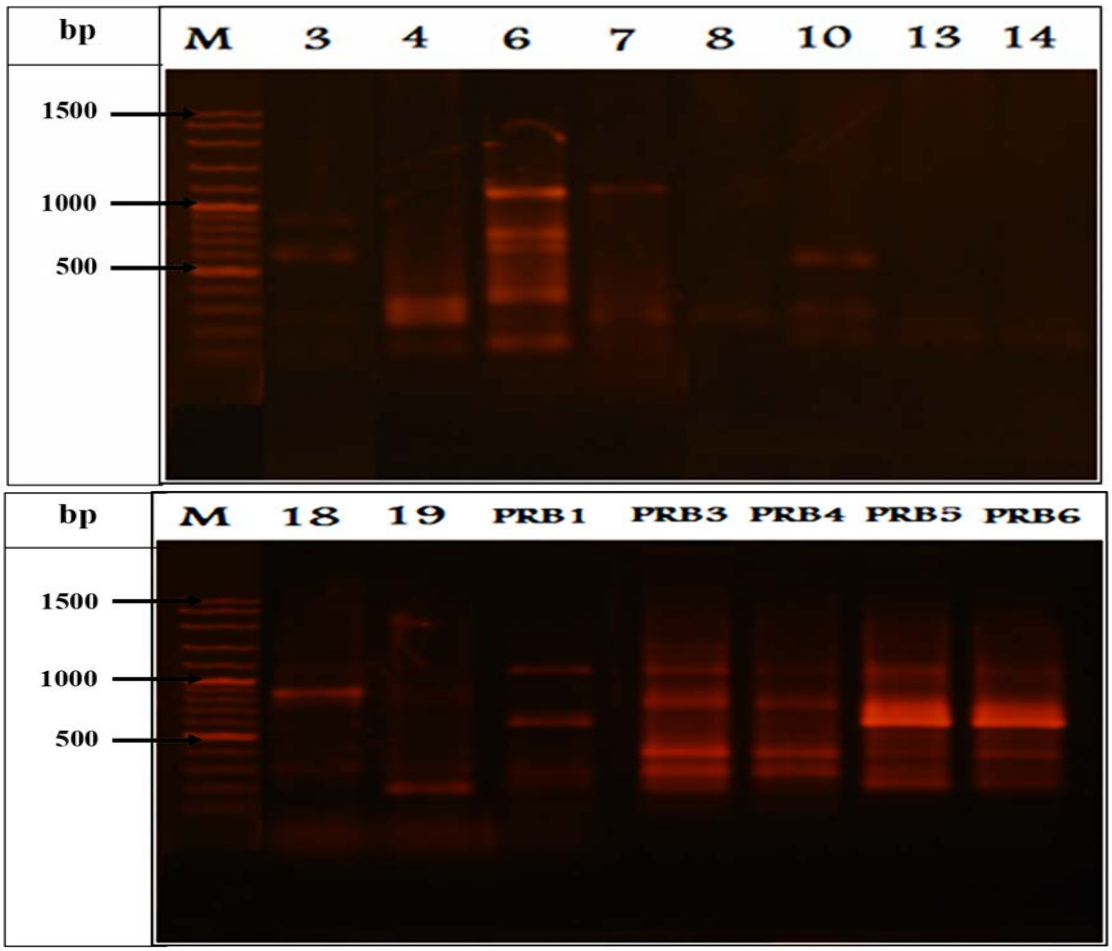
(d)



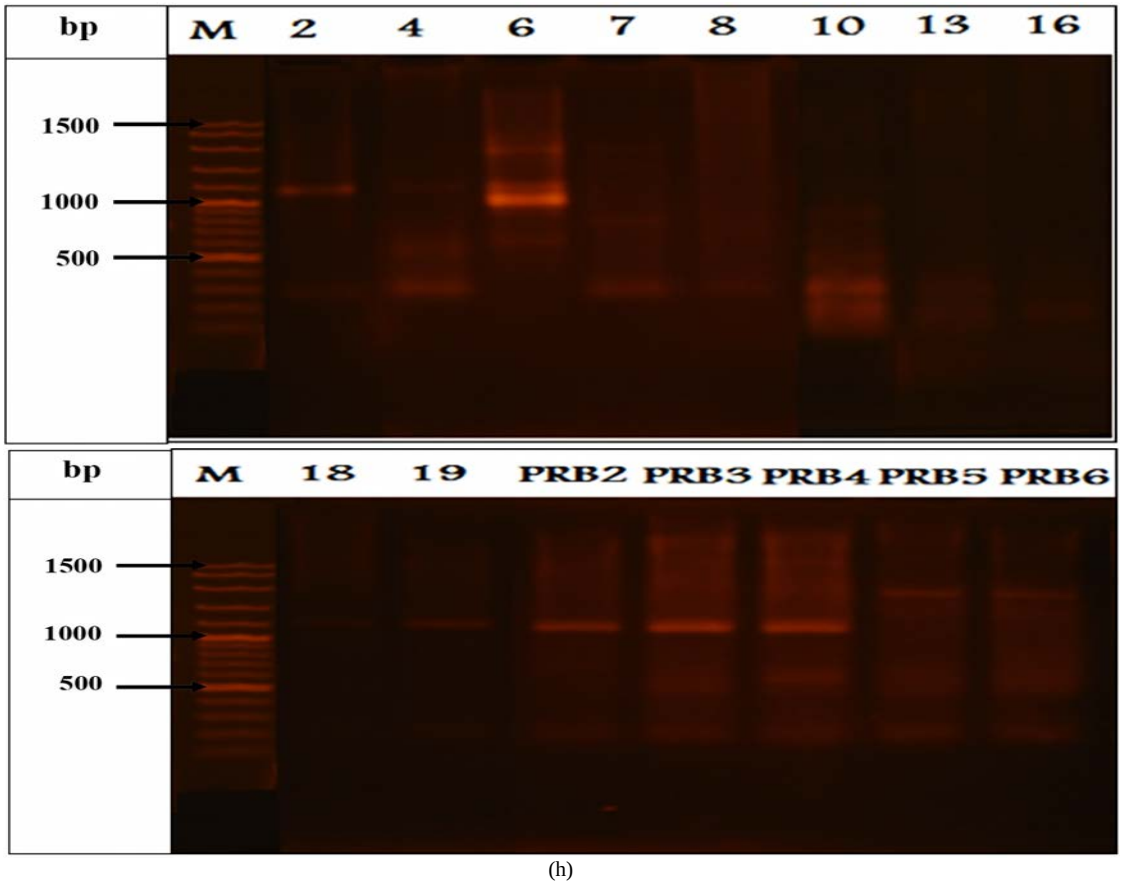
(e)



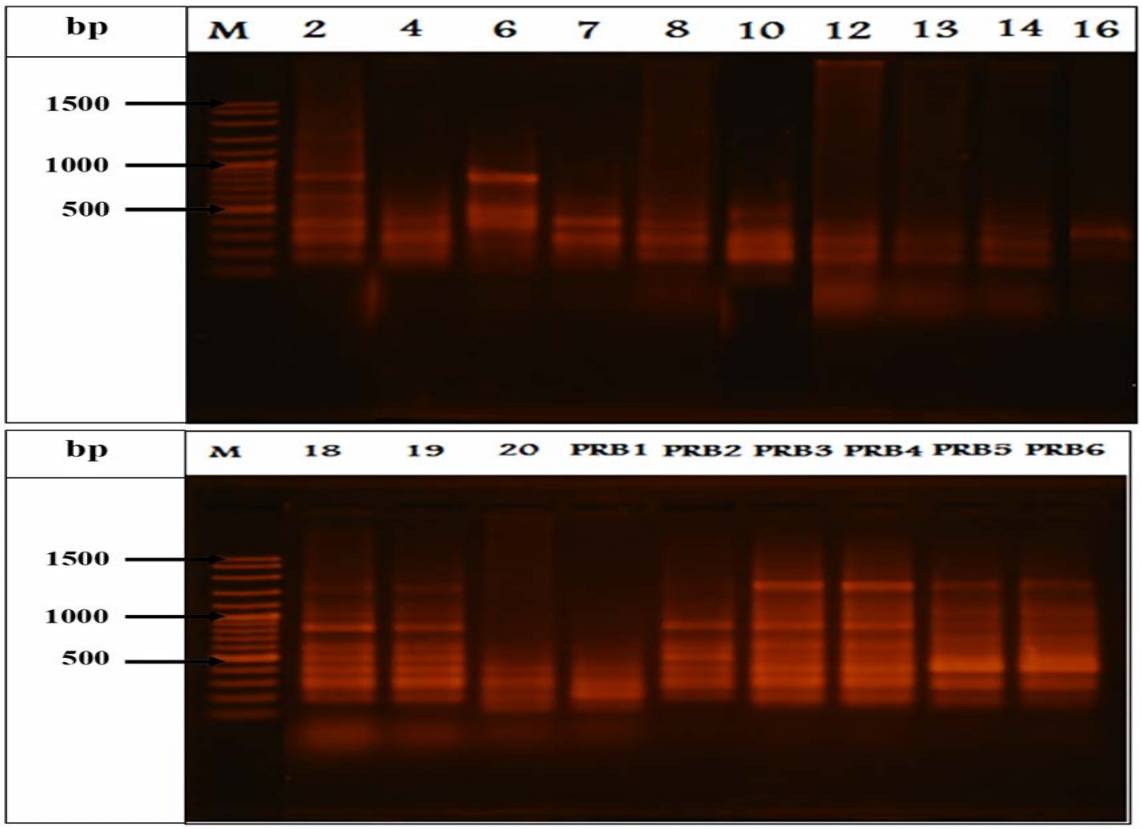
(f)


bp
M 3 4 6 7 8 10 13 14
1500
1000
500
bp
M 18 19 PRB1 PRB3 PRB4 PRB5 PRB6
1500
1000
500

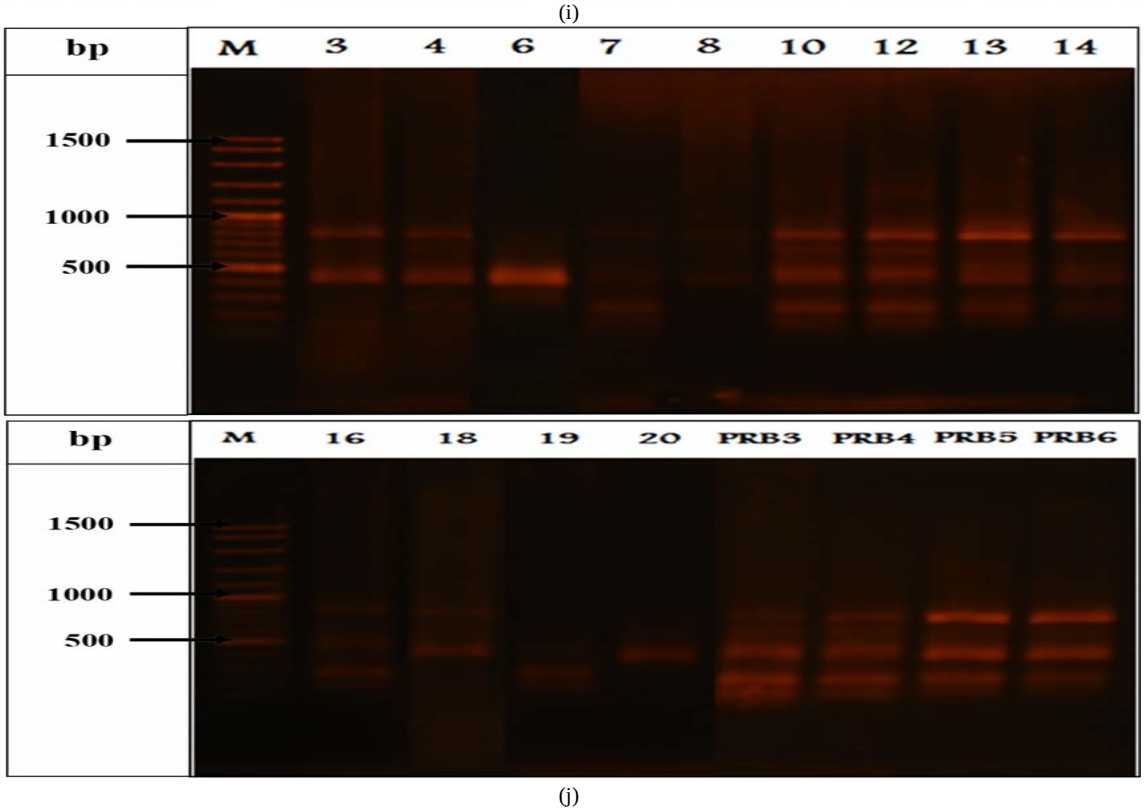
(g)


bp
M 2 4 6 7 8 10 13 16
1500
1000
500
bp
M 18 19 PRB2 PRB3 PRB4 PRB5 PRB6
1500
1000
500

(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)**	**∑ 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	1 200	Prb3
						900	Mdr19
8	OPE 8	7	7	100%	2	1 300	Prb5
						500	Mdr4
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.

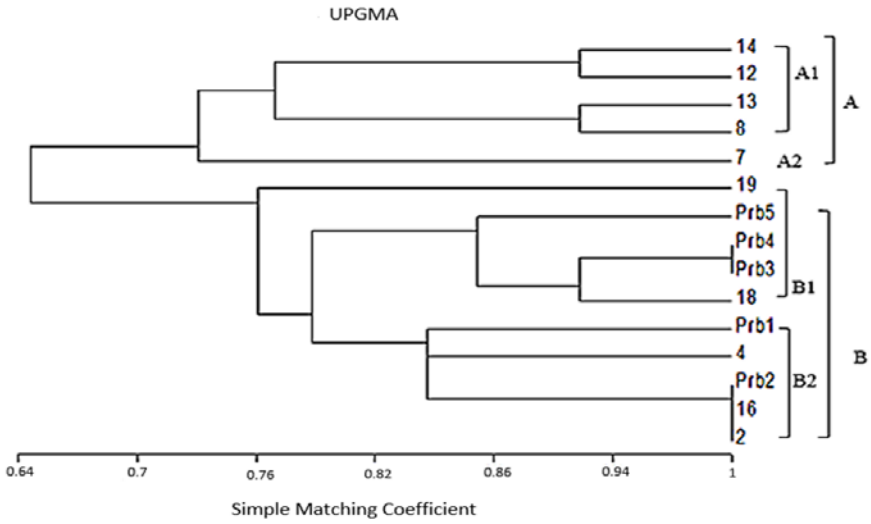


Figure 2. The dendrogram showing the connection amongst 15 accessions of *P. lunatus* referring to RAPD marker

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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