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Genetic evaluation of F2 and F3 interspecific hybrids of mung bean (*Vigna radiata* L. Wilczek) using retrotransposon-based insertion polymorphism and sequence-related amplified polymorphism markers

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ABSTRACT Mung bean (*Vigna radiata* L. Wilczek) is a self-pollinating and indispensable pulse crop in Indonesia. While low yield productivity is a major concern, genetic improvement is possible through interspecific hybridization. However, interspecific hybridization is relatively infrequent and produces low recombination exchanges, significantly limiting crop breeding efficiency. Thus, a comprehensive study is needed of the selection and genetic diversity evaluation of progenies in advanced generations derived from interspecific hybridization using a specific molecular marker. This study aims to confirm the heterozygosity in the F2 population and assess the genetic diversity in F3 mung bean populations resulting from interspecific hybridization between the mung bean and common bean. We designed the retrotransposon-based insertion polymorphism (RBIP) marker by identifying the syntenic regions in the flanking sequences of retrotransposon insertion in common bean and mung bean. The RBIP marker can be applied to distinguish the heterozygote progenies from the homozygote progenies. Six combinations of sequence-related amplified polymorphism (SRAP) primers were used in the genotyping of F3 mung bean progenies. The SRAP marker showed a high degree of polymorphism of up to 100%, while high genetic variation was observed within the population (71%) of mung bean progenies. The F3.4 population had the greatest number of genotypes and displayed the highest number of effective alleles, private alleles, and percentage of polymorphic loci, suggesting the existence of high genetic diversity within this population. These genetic diversity data are exceptionally critical for future genetic research since it has potentially high yield production. The genomic and marker-assisted selection studies will support the major goals of the mung bean breeding program.

KEYWORDS Genetic differentiation; Heterozygosity; Intergeneric hybrids; Mung bean breeding; RBIP marker

1. Introduction

Mung bean (*Vigna radiata* L. Wilczek) is an annual and pulse crop, self-pollinated, and important legume crop in Asia (Lin et al. 2020). Mung bean are rich in vitamins, fibers, minerals, carbohydrates, proteins, and can be used as potential crop for mitigation of malnutrition (Ganesan and Xu 2018), and commonly used as supplemental dishes and healthy food in Indonesia (Novidiyanto et al. 2019). Mung bean is fairly resistant to abiotic stresses, but productivity has remained low due to biotic challenges as well as a lack of variation with significant yield potential, big seed size, and high weight per seed. The production potential of an Indonesian mung bean is up to 2.5 ton/ha, with an average productivity of about 0.9 ton/ha (Taufiq and Kristiono 2016). Cultivated mung bean had a narrow genetic basis represent low genetic variability in primary gene pool (Noble et al. 2018; Fatmawati et al. 2021).

To accelerate the genetic improvement of mung bean, interspecific hybridization approach can be utilized for creating interspecific recombinant and produce superior genotypes through mung bean breeding project. Interspecific hybridization is indispensable method for breeding program (Zhan et al. 2017). Interspecific hybridization has been reported for genetic speciation, genome evolution, genetic diversity, introgression novel gene as well as improve adaptability to new environment, increasing yield, and essential nutrition to support biofortified breeding program (Abbas et al. 2015; Zhang et al. 2016; Yu et al. 2021). The common bean (*Phaseolus vulgaris* L.) and mung bean, belonged to the Fabaceae family, separated

from a common ancestor between 4.9 and 8 million years ago (Lavin et al. 2005). Thus, the mung bean-common bean linkage group is generally preserved, and synteny analysis of mung bean unigene sequences indicated gene function similarities with common bean (McClean et al. 2010).

Interspecific hybridization in mung bean has been reported to be effective (Abbas et al. 2015; Pandiyan et al. 2020), but no studies of interspecific hybridization with common bean have been reported. Although segregation distortion has been reported in the majority of F2 progenies derived from interspecific (Toyomoto et al. 2019; Shehzad et al. 2021), we were able to successfully evaluate the F2 interspecific progenies from this crossing and maintain the elite genotypes through pedigree selection for F3 generation. To validate the genetic constitution of interspecific hybrids in mung bean, DNA marker is preferable to be used for characterization of the genetic background on material tested, compared to morphological characterization since it is laborious task and often affected by environmental condition (Sormin et al. 2021). In addition, DNA marker can be applied to assess genetic diversity, evolution, and phylogeny, investigate heterosis, identify haploid/diploid plants and cultivar genotyping, and marker assisted selection (Nadeem et al. 2018).

Transposable element in particular retrotransposon is relatively high abundant in mung bean genome (Kang et al. 2014). This mobile element is well organized in chromosomes and inserted into multiple gene loci (Setiawan et al. 2020). Mung bean genome comprise of repetitive sequences (50.1%), in which 36.5% consist of long terminal repeat (LTR) retrotransposon (Kang et al. 2014). Interspecific hybridization may influence the activation of mobile elements in hybrids, potentially leading to plant speciation and insertion of specific gene of interest (Glombik et al. 2020). Thus, transposable elements are useful to be utilized as molecular marker due to their abundance in plant genome. Transposable element-based markers such as inter-retrotransposon amplified polymorphism (IRAP), miniature-inverted repeat transposable element (MITE), inter-SINE amplified polymorphism (ISAP), retrotransposon-based insertion polymorphism (RBIP) has been used in plant genotyping of melon (Sormin et al. 2021), identification of somaclonal variation in date palm (Mirani et al. 2020), and genetic diversity assessment in mango (Nashima et al. 2017).

Our previous works confirmed that the F2 population of mung bean derived from interspecific hybrids was verified as genuine hybrids by employing a dominant IRAP marker (Fatmawati et al. 2021). However, this marker could not identify heterozygote genotypes, and a codominant marker is prefered for analyzing the genetic constitution of these hybrids. In addition, interspecific hybridization is typically followed by whole-genome or fragmented DNA duplication to ensure the stability of the genuine hybrids (Glombik et al. 2020). Therefore, the use of codominant markers is critical for the identification of genotypes containing the novel genetic recombination from both parents in the advanced generation. Even though single sequence repeat (SSR) is a codominant marker that has been used to assess genetic diversity in mung bean accessions (Kaur et al. 2018) and the hybrid vigor of mung bean F1 hybrids (Sorajjapinun et al. 2012), but it required many primer-sets and was only appropriate for progenies derived from intraspecific hybridization in which their parental lines shared similar genomic constitutions. The retrotransposon based codominant marker can be designed by determining different allelic states at certain loci from both parental lines utilized for interspecific hybridization by identifying the flanking sequence of retrotransposon insertion. The RBIP is a PCR-based marker that can detect transposable element insertions in plant genomes at a specific locus and provide an accurate DNA profile. The genomic DNA can be amplified using LTR and flanking region-specific primer sets. In addition, RBIP has been used in plant genetic studies (Kim et al. 2012; Schulman et al. 2012; Nashima et al. 2017).

After confirming and selecting the F2 was genuine hybrids, we developed an F3 population by selecting elite genotypes from F2 population through pedigree selection. Genetic variation in the F3 population is critical for the mung bean breeding effort, particularly when determining advanced elite genotypes and/or choosing future parents (Baenziger et al. 2011). The genetic diversity of the F3 population can be analyzed using sequence-related amplified polymorphism (SRAP) (Purwantoro et al. 2023). SRAP is a PCR-based marker, consist of 17 or 18 nucleotides that amplified the open reading frames (Li and Quiros 2001). SRAP is dominant marker which has been successfully applied to investigate the genetic diversity in Indian garlic (Benke et al. 2021) and mung bean (Aneja et al. 2013). This study aimed to identify the flanking genomic sequences from both the mung bean and common bean genomes and design the RBIP marker from a highly conserved region to investigate heterozygosity in the F2 population. This work also aims to assess genetic diversity in F3 mung bean populations resulting from interspecific hybridization between the mung bean and common bean using SRAP marker.

2. Materials and Methods

2.1. Plant materials

The F2 population of interspecific hybrids that used by Fatmawati et al. (2021) was utilized to study their genetic constitutions using the RBIP marker. Four elite genotypes of mung bean selected from F2 generation were used for F3 main population. Each elite genotype consisted of 16 plants. These 64 genotypes were derived from interspecific hybridization [mung bean landrace 'lokal malang' (*Vigna radiata* L. Wilczek) × common bean cultivar 'Lebat-3' (*Phaseolus vulgaris* L.)]. The plants were cultivated in Research Station of Banguntapan, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta from October 2020 until April 2021.

TABLE 1 Marker utility of SRAP in F3 interspecific hybrid of mung bean.

Primer combination	Forward primer (me)	Reverse Primer (em)	Band size (bp)	TPL	TAL	DP (%)
me7em8	TGAGTCCAAACCGGACG	GACTGCGTACGAATTCAC	100-2000	25	25	100
me7em9	TGAGTCCAAAC GGACG	GACTGCGTACGAATTCAG	100-2000	25	25	100
me7em10	TGAGTCCAAACCGGACG	GACTGCGTACGAATTCAT	100-2000	28	28	100
me9em9	TGAGTCCAAACCGGAGG	GACTGCGTACGAATTCAG	100-1750	25	25	100
me9em10	TGAGTCCAAACCGGAGG	GACTGCGTACGAATTCAT	100-1000	10	10	100
me10em10	TGAGTCCAAACCGGAAA	GACTGCGTACGAATTCAT	150-750	7	9	77.78
Total				120	122	
Mean				20.00	20.33	96.29

Remark: TPL = Total polymorphic loci; TAL = Total amplified loci; DP = Degree of polymorphism.

2.2. Isolation and quantitation of genomic DNA

Total DNA was isolated from mung bean leaves using a modified CTAB (hexadecyltrimethylammonium bromide) as described in Dharajiya et al. (2017). NanoDrop (2000c Spectrometer, Thermo Scientific) was used to quantify DNA samples. Then, DNA were diluted using nuclease free water (NFW) into working solution 25 (ng/µL).

2.3. Synteny analysis of mung bean-common bean and RBIP marker design

The bacteria artificial chromosome (BAC) clone of *Phaseolus vulgaris* PVGBa_61E16, the accession of genbank ID GU215957.1 was retrieved from the National Center for Biotechnology Information (NCBI). The LTR sequence and Ty1/Copia retrotransposon and the motif of the sequence was detected and confirmed by LTR finder (Xu and Wang 2007) and Conserved Domain Database (CDD) of NCBI (Lu et al. 2020), respectively. The synteny analysis of the flanking genomic sequence of retrotransposon between mung bean and common bean was conducted using a dotlet (Junier and Pagni 2000). The flanking sequence of 3′ LTR retrotransposon from *P. vulgaris* was subjected to Dotlet JS (https://dotlet.vital-it.ch) against *V. radiata* genome sequence (LJIH01000004.1:c168862-160236) to identify the synteny region. Multiple sequence alignment (MSA) of syntenic region from the flanking genomic sequence was generated using ClustalW embedded in BioEdit. The RBIP primers were designed from the highly conserved region to obtain PCR products with different size between *P. vulgaris* and *V. radiata* using FastPCR (Kalendar et al. 2017). The flanking region was amplified by PCR using primer pairs 5′-ACCATTTAAGCCCAAGGTTCAACCTCA-3′ and 5′-GAGACTTTCCTCTGCATATGAAC-3′.

2.4. PCR amplification

The amplification of DNA was carried out using T100™ thermal cycler (Bio-Rad, USA). The PCR reaction of RBIP and SRAP was consisted of 50 ng of gDNA, 0.2 mM dNTPs, 0.2 µM primer, 1X GoTaq® Green Master Mix (Promega, USA), 1.25 U/µL GoTaq® polymerase, and added with NFW into final volume 12.5 µL. The amplification of RBIP condition consisted of pre-denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, with an extension at 72 °C for 2 min, and the final extension at 72 °C for 10 min. The DNA amplification using IRAP marker was conducted in accordance to Fatmawati et al. (2021). The PCR condition of SRAP were conducted in accordance with Li and Quiros (2001). In brief, pre-denaturation at 94 °C for 2 min, annealing at 35 °C for 1 min, and extension at 72 °C, 1 min are the first five cycles. In additional 35 cycles, the annealing temperature was raised to 50 °C for 1 min, followed by an 8-min extension at 72 °C. The SRAP primer sequences are listed in Table 1 as described in Uzun et al. (2009).

2.5. Data analysis

The amplified bands were scored as 1 if they were present and 0 if they were not to generated binary data. NTSYS-PC software was used to do the cluster analysis (Rohlf 2009). The binary data were subjected to genetic similarity matrix using simple matching on the similarity of quantitative data (SIMQUAL) program. The mean of the unweighted pair group method with arithmetic average (UPGMA) technique was used to create a dendrogram. Number of distinct alleles (Na), number of effective alleles (Ne), expected heterozygosity (He), number of loci with private allele (Pa), and percentage of polymorphic loci (PPL) were used to compute genetic indices. The genetic distance between mung bean genotypes was used to perform principal coordinate analysis (PCoA). The analysis of molecular variance (AMOVA) and PCoA were carried out using the GenAIEx software version 6.5 (Peakall and Smouse 2012).

3. Results and Discussion

3.1. RBIP marker design and genetic confirmation of F2 hybrids

An LTR retrotransposon was detected from BAC clone of *Phaseolus vulgaris* (GU215957.1) using LTR Finder (Xu and Wang 2007). The sequence length of this retrotransposon was 4200 bp, and the lengths of its 5 and 3 LTRs were 320 and 317 bp, respectively. This retrotransposon shared 97.20% of LTR similarity and target site duplication was

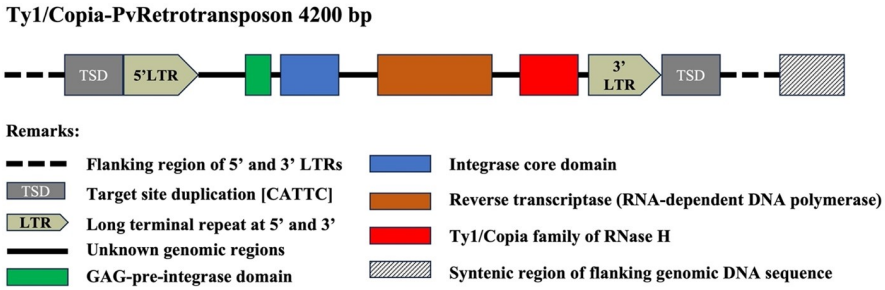


FIGURE 1 Ty1/Copia retrotransposon structure identified in *Phaseolus vulgaris* containing flanked DNA sequence that high synteny to *Vigna radiata*.

marked by CATTC at both the 5′ and 3′ end of LTR. This LTR retrotransposon was classified as Ty1/Copia retrotransposon based on Conserved Domain Database (CDD) analysis (Figure 1). This Ty1/Copia sequence was made up of GAG, Integrase, Reverse Transcriptase, and RNAse HI sequences with lengths of 215, 338, 743, and 401 bp, respectively. The dot plot result revealed that the flanking sequence of the Ty1/Copia retrotransposon in *Phaseolus vulgaris* shared the conserved syntenic region with *Vigna radiata* (Figure 2). Based on synteny analysis of unigene sequences, mung bean and common bean was comparable with their gene functions, and the linkage groups among them are largely conserved (McClean et al. 2010). In addition, mung bean is closely linked to common bean which separated from a common ancestor (Lavin et al. 2005; Stefanović et al. 2009).

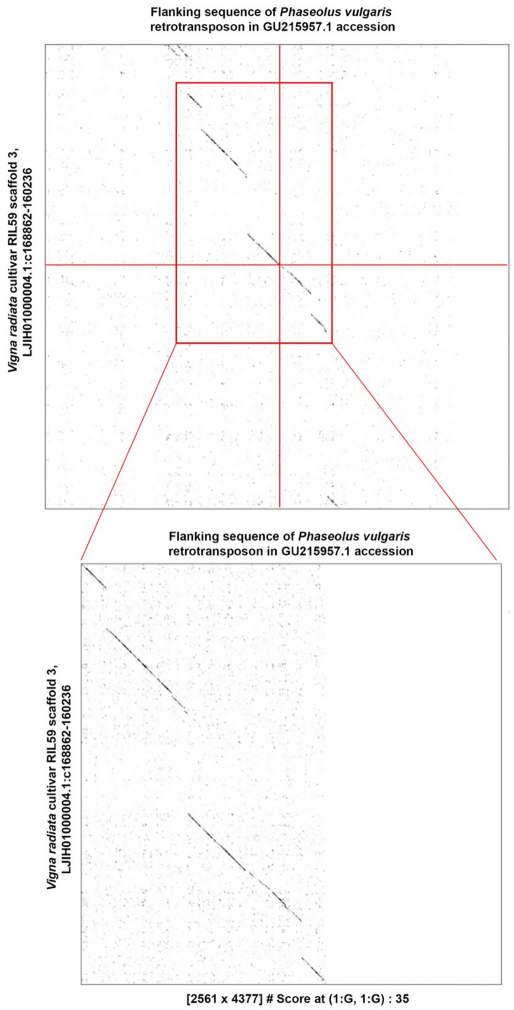


FIGURE 2 Dot plot analysis of *Phaseolus vulgaris* and *Vigna radiata* genomic sequences synteny region that flanked at the 3′ LTR of Ty1/Copia retrotransposon.

The MSA of flanking genomic region was analyzed using Bioedit and the primers were designed from highly conserved syntenic region to obtain the PCR product with different size from both plant species (Figure 3). The PCR amplification from both of 'lokal malang' (female parent) and 'Lebat-3' (male parent) with RBIP primers generated distinct amplicon size around 1300 bp and 900 bp, respectively (Figure 4b). The heterozygote progenies can be clearly recognized from the homozygote progenies because they inherit alleles from both parents (Figure 4b). The homozygous progenies only had a single band that was identical to the female parent, such as genotypes of 43, 49, and 50. In contrast, IRAP, a dominant marker, was not unable to distinguish the heterozygotes (Figure 4a).

The successful hybridization of two parental lines resulted in a novel genetic recombination in their progeny. Genetic recombination determines population diversity and generates unique allele combinations (Fernandes et al. 2018). Recombination rates consider to be different among species, populations, individuals, sex, chromosomes, and intrachromosomal locations (Dreissig et al. 2019). The successful genetic recombination determines by the successful hybridization and fertilization. However, the interspecific hybridization is relatively infrequent, produces low recombination exchanges, significantly limiting crop breeding efficiency (Shen et al. 2021). Therefore, comprehensive identification of progenies resulting from interspecific hybridization should be carried out using genetic markers. In this results, the RBIP marker used in this study was confirmed and supported that the progenies are genuine hybrids derived from interspecific hybridization between mung bean and common bean that has been validate by Fatmawati et al. (2021) using IRAP

marker and the progeny had a distinct morphological character in seed size and seed coat color (Fatmawati et al. 2021; Fatmawati 2022).

3.2. SRAP marker analysis of F3 interspecific hybrid of mung bean

In this study, we demonstrated the application of SRAP marker to characterize genetic diversity of F3 mung bean population. High level polymorphism was observed when SRAP marker applied in mung bean genotyping. Six combination SRAP primers has been successfully amplified PCR products to characterize genetic diversity in all mung bean genotypes. All primers produced 122 of total amplified loci out of which 120 loci were polymorphic (Table 1). The amplicon sizes and degree of polymorphism were varied from 100 to 2000 bp and 77.78% to 100.00%, respectively. These results imply that all these SRAP primers were highly effective for mung bean genotyping. The use of molecular markers is essential, especially when assessing the genetic diversity of a population produced via inter-

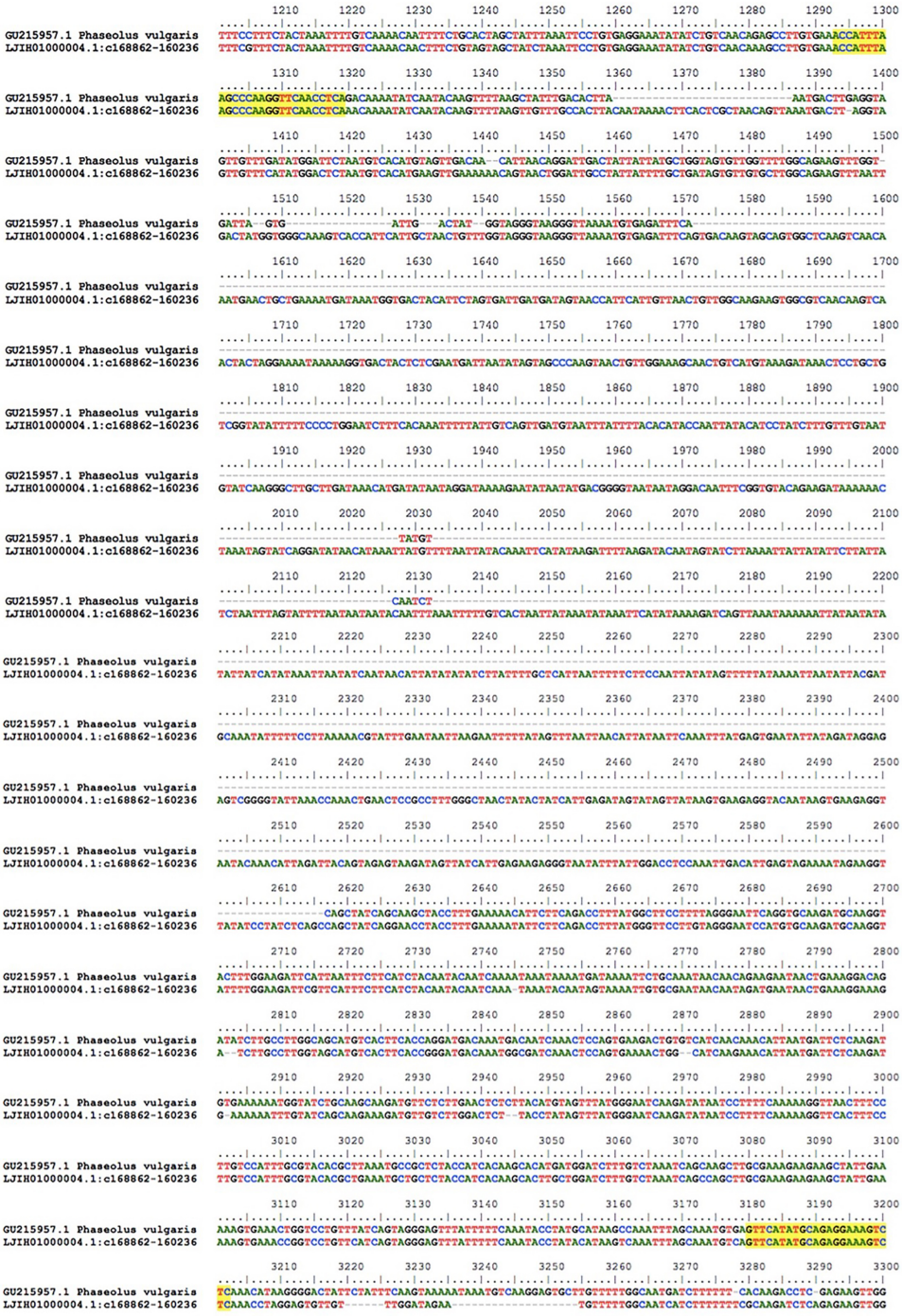
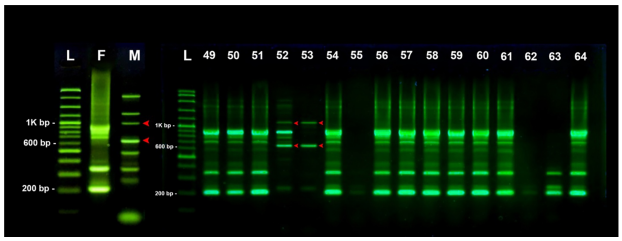
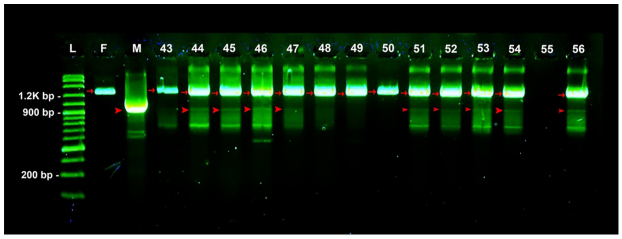


FIGURE 3 Multiple sequence alignment of flanking genomic region of *Phaseolus vulgaris* and *Vigna radiata*. Yellow colors depict the primer position.



(a)



(b)

FIGURE 4 DNA profile of F2 interspecific hybrids amplified from using IRAP and RBIP. IRAP banding pattern in F2 interspecific hybrids (a), RBIP banding pattern in F2 interspecific hybrids (b). Red arrow heads represent specific alleles from common bean [male parent (M)] inherited in progenies. The white arrow heads represent the progenies that shared a particular amplicon with the female parent (F).

specific hybridization. Mung bean lacks a barier in which to cross with closely related species (Pandiyan et al. 2020). Furthermore, rigorous research should be carried out utilizing molecular markers to determine genetic variety in advanced genotypes. Thus, using SRAP marker, we confirmed that this F3 population of was genetically varied and exhibited high degree of polymorphism.

3.3. Genetic diversity of F3 mung bean population

The values of Na and Ne ranged from 0.967 to 1.336 and 1.127 to 1.148, respectively (Table 2). When compared to other populations, the F3.2 population has the most distinct alleles. The He value ranged from 0.089 to 0.117, while the PPL value ranged from 45.08 to 63.11 percent. The F3.2 population has the highest He and PPL values. The Pa value for the entire population ranged from 2 to 14 bands. Both the F3.2 and F3.4 populations have a high Pa value, with 13 and 14 bands, respectively. These findings indicate that the genotypes in the F3.2 and F3.4 populations have a significant genetic diversity when compared to other populations. This finding is corroborated by the AMOVA, which shows that genetic variation is prevalent within populations (Table 3). According to the AMOVA data, there was 71% variance within the population and 29% variation among populations (Table 3). The variance was calculated using 999 permutations. With a moderate degree of genetic diversity, population variation is considerably different (29%). This finding is confirmed by the large number of total private alleles produced in all populations (34), of which 13 and 14 private alleles occurred in F3.2 (38.23%) and F3.4 (41.17%), respectively (Table 2). The selection of characteristics linked to yield components and seed coat colors in F2 population of mung bean may have resulted in high diversity within the population.

The F3.4 population presented remarkable genetic differentiation in the number of private allele (Table 2). The reason for the increased genetic diversity among genotypes of the F3.4 population might be because it is free of segregation distortion in F2 and its progenies inherit the alleles from their male parent (common bean). The private allele denotes that the allele exists solely in one population. The F3.4 population comprised 14 private alleles compared to F3.1 and F3.3, which had 5 and 2 private alleles, respectively, showing the considerable genetic diversity that occurred among the F3.4 genotypes. Private allele data provide valuable information on the unique genetic variety at specific loci, as well as identifying highly diverse genotypes that might be used as parental lines in plant breeding programs to enhance allele diversity in the population (Salem and Sallam 2016). The allelic pattern and genetic diversity indices were beneficial in determining genetic variation in each population. Even though the three populations had distinct diversity, the F3.4 had the

TABLE 2 The average of various genetic factors in each mung bean population.

Population	Na	Ne	He	PPL (%)	Pa
F3.1	0.967±0.088	1.146±0.025	0.091±0.014	45.08	5
F3.2	1.336±0.082	1.170±0.022	0.117±0.013	63.11	13
F3.3	1.016±0.089	1.127±0.019	0.089±0.012	48.36	2
F3.4	1.131±0.085	1.148±0.021	0.102±0.012	51.64	14
Mean	1.113±0.043	1.148±0.011	0.100±0.006	52.05	8.5

TABLE 3 The AMOVA analysis using 6 SRAP primer combinations of the genetic differentiation among and within four population of 64 mung bean genotypes.

Source	df	SS	MS	Est. Var	% Var	P-value
Among population	3	193.266	64.422	3.488	29%	0.001
Within population	60	516.438	8.607	8.607	71%	
Total	63	709.703		12.096	100%	

Remark: df = degree of freedom; SS = sum of square; MS = mean of square; Est. Var = estimated variance; % Var = percentage of variation.

most genetic diversity. Understanding genetic variation in the interspecific hybridization of mung bean breeding program will help in identifying superior genotype for further selection in advance generation.

3.4. Cluster analysis of F3 mung bean population

Understanding the genetic diversity of this population requires the identification of F3 population structure. UPGMA clustering of simple matching similarity data from all six marker combinations split 64 F3 genotypes into six different groups (Figure 5). The similarity coefficient was varied from 0.75 to 0.98. There were 39 individuals in Cluster I. On the other hand, Clusters II, III, IV, V and VI had 2 members, 2 members, 6 members, 16 members, and 1 member, respectively. This finding suggests that ISAP is a rather good marker for identifying genetic diversity in an interspecific hybrid F3 population. A PCoA biplot analysis validated the UPGMA clustering result, which divided the 64 mung bean genotypes into four groups (Figure 6). All 64 genotypes of F3 mung bean population were divided into four quadrants. Quadrant I included the genotypes of the F3.1 population. The predominance of F3.2 and F3.3 genotypes were found in quadrant II. Several genotypes of F3.2 and F3.4 populations were found in quadrant III. The most of F3.4 populations were found in quadrant IV. This result implies that the F3.4 population was distinguished from other populations, which is corroborated by the UPGMA dendrogram result (Figure 5). The findings of the dendrogram analysis (UPGMA) agreed with the results of the PCoA biplot (Figures 5 and 6). This result implies that the F3 population structure that has been selected from F2 generation had a distinct genetic background which supported by the phenotypic variation of seed coat color and yield traits (Fatmawati 2022). Furthermore, knowing population structure is critical for identifying marker-associated characteristics using genome-wide association studies (GWAS) (Eltaher et al. 2018). As a result, before doing GWAS to find a proper correlation between a characteristic of interest and markers that might lead to the identification of underlying genes, the first stage is to evaluate the population structure.

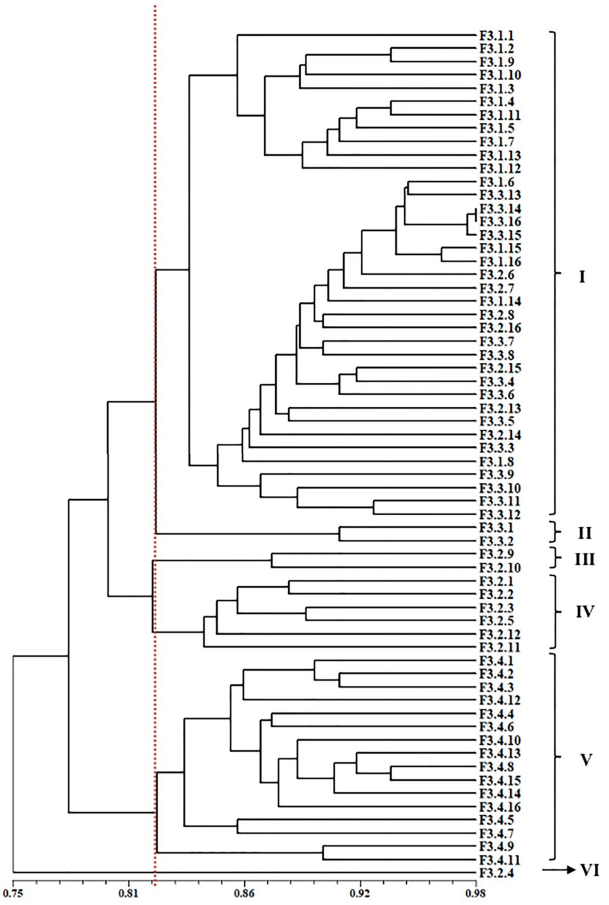


FIGURE 5 Dendrogram of 64 genotypes of mung bean in F3 population based on SRAP markers which separated the genotypes into 6 clusters with the genetic similarity coefficient is 0.82.

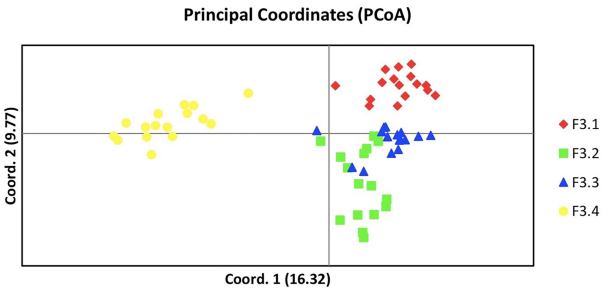


FIGURE 6 Principle coordinate analysis (PCoA) of 64 genotypes of mung bean in F3 population based on SRAP markers.

4. Conclusions

In conclusion, the RBIP marker can be used to differentiate the heterozygote progenies in F2 population of interspecific hybrids. The F3.4 genotype population had the highest number of private alleles, polymorphic loci percentage, and effective alleles. Despite their tremendous selection, the elite genotypes were genetically diverse. This mung bean population structure and genetic diversity information is critical for future genetic studies such as GWAS and marker-assisted selection studies for high yield potential and nutritional value of mung bean.

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Authors' contributions

YF and I performed the experiment and analyzed the data for RBIP and SRAP markers in F2 and F3 generation. ABS designed the research, performed bioinformatic analysis, analyzed and interpreted the data, and wrote the manuscript. AP and DWR designed the research and

review the final manuscript. CHT performed bioinformatic analysis, examine the data, and review the final manuscript.

Competing interests

All authors declare that there are not any conflicts of interest.

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