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Posted Date: 8 August 2023

doi: 10.20944/preprints202308.0658.v1

Keywords: Mungbean, Vigna, Whole genome sequence, SSR markers, Principal co-ordinate analysis, Genetic diversity

*Article*

# Validation and Cross-Species Transferability of SSR Markers Developed from Whole Genome Sequence (WGS) of Mungbean (*Vigna radiata* L. Wilczek) and Their Application of Population Genetics in *Vigna* Species

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**Abstract:** The genus *Vigna* is penta-tropical having more than 200 species with many desirable economically important traits. The aim of study was to validate the *in-silico* polymorphism of whole genome sequence developed mungbean specific SSR markers and their transferability among the different *Vigna* species. The present study utilized a set of 200 SSR markers developed from whole genome sequence of mungbean and validated using a diversity panel of 25 accessions which belongs to 13 *Vigna* species. Out of 200 SSR markers, 130 markers (65%) were polymorphic across the various *Vigna* species and the number of alleles amplified varied between 7 to24. The SSR markers showed more than 90 percent transferability across the different *Vigna* species accessions. Based on allelic data, the 25 *Vigna* accessions grouped into three clusters based upon the unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree. The principal co-ordinates analysis (PCA) biplot graph and UPGMA based neighbor joining clustering diagram showed similar pattern of *Vigna* accessions distribution. The population structure assessment has grouped the cultivated and wild species accessions into two sub-population. The estimated marker parameters such as polymorphic information content (0.09 – 0.84), marker index (0.091 – 3.342) and effective multiplex ratio (1.0 – 4.0) suggested their adequacy in several genetic studies such as parental selection, hybrid testing, genetic mapping and marker aided breeding programmes for genetic enhancement of species belonging to the *Vigna* genus.

**Keywords:** mungbean; *Vigna*; whole genome sequence; SSR markers; principal co-ordinate analysis; genetic diversity

## 1. Introduction

Among the legumes, *Vigna* is an agriculturally important taxon. The genus *Vigna* belonging to subgenus *Ceratotropis*, tribe Phasleoleae, family Fabaceae, include more than 150 *Vigna*species. The majority of members belonging to *Vigna* are wild species from Asia and Africa continents [1,2]. The domesticated members of *Vigna* include 10 species such as mungbean (*V. radiata* L. Wilczek), ricebean (*V. umbellata* (Thunb.) Ohwi & Ohashi), adzuki bean (*V. angularis* (Willd.) Ohwi & Ohashi), urdbean

 doi:10.20944/preprints202308.0658.v1



(*V. mungo* L. Hepper), moth bean (*V. aconitifolia* (Jacq.) Marechal), cowpea (*V. unguiculata* L. Walpers), creole bean (*V. reflex-pilosa*) and Bambara groundnuts (*V. subterranean* (L.) Verdc.) [3,4]. Except creole bean (2n=2x=44, tetraploid), all these cultivated species are diploid in nature (2n=2x=22). The subgenus *Ceratotropis* is further grouped in three divisions namely *Ceratotropis* (mungbean,blackgram), *Aconitifoliae* (mothbean) and *Angularis* (adzuki bean and creole bean) [5]. This subgenus is the dockyard of desirable adaptive genes for evolution of climate resilient *Vigna* cultigens. In India, enormous diversity of *Vigna* species occur in the Western Ghats (Gujrat, Maharashtra, Nilgiris, Karnataka, Kerala, Tamil Nadu), Eastern Ghats (Odisha, West Bengal), Central plateu (Chhatisgarh, Madhya Pradesh, Maharashtra); North Western Himalayas (parts of Uttaranchal and Himachal Pradesh) [6].

Among *Vigna*, Mungbean (*Vignaradiata* L. Wilczek) is a major pulse popularly referred as green gram, golden gram, oregon pea, chickasawpea and mung [7]. It is autogamous crop with 2n=2x= 22 chromosomes which spanned about 574 Megabasepairgenetic material [8]. The small genome size makes it a suitable model crop for studying the evolutionary and genetic diversity studies [9]. It contains ample amount of easily digestible protein which is very much useful in addressing health problems such as diabetes and malnutrition. The haulms of mungbean is generally used in animal husbandry for feeding to domesticated animals as it does not have any negative effects on animal health [10,11].The mungbean seeds are free of anti-nutritional factors (trypsin inhibitors, tannin, phytohemagglutinin, etc.,) [12]. Apart from its use as food and feed, mungbean is sought-after for its N-fixing ability in soil through symbiotic association with *Rhizobium* spp., *Bradyrhizobium* bacteria in their root nodules.The nitrogen fixation mechanism enhances the soil fertility which in turn increases the financial condition of small and marginal farmers. The crop is also acclaimed for its ability to perform well in marginal lands, under limited moisture and essential mineral elements available in surroundings mainly owing to their symbiotic association with N-fixingRhizobium bacteria and arbuscularmycorrhizal (AM) fungi that help to ameliorate themineral elements acquisition from humus and crop-establishment in such lands [13].

The crop is grown popularly in South and North America, West Indies, Australia, Asia and Tropical and Subtropical Africa. Currently, mungbean is grown on about 7.3 million hectares mainly in Asia across different seasons (spring, summer, *kharif*and*rabi*) and the cultivation is expanding into Africa and Australia [14,15]. The worldwide mungbean harvest is 5.3 million tons and 51% is from India after Myanmar and China [15]. In India, mungbean occupies 4.32 million hectares area mainly in Rajasthan, Maharashtra, Andhra Pradesh, Karnataka, Odisha, Uttar Pradesh and Bihar and resulted into a harvest of 2.17 million tons [16]. At global level, India produced about 54% of total mungbean production with 65% acreage of world mungbean acreage [17]. The Asian continent exhibited a knee-high average productivity of mungbean due to the inherently low yielding potential of the cultivars and their susceptibility to fungal, bacterial, viral and other foliar diseases [18,19]. However, in the present scenario themungbean cultivars which matures in less time, photo-insensitive, stable and high level of resistance against disease and insect pests and high yielding in nature provides a chance to cultivate mungbean as catch crop in cereal cropping system (rice-wheat-mungbean). The diverse edapho-climatic conditions of India are well suited for sustainable food production and food security [20]. Further expansion of mungbean cultivation is linked to pace of genetic improvement which depends upon genetic and genomic resources. Mungbean is lagging in genomics research and application of genomics assisted breeding techniques than other legume crops. Till date, 18 genetic linkage maps are available in mungbean based on RFLP, RAPD, STS, SSR from mungbean and other species [21]. Several researchers [22,23,24] developed mungbean specific DNA markers *i.e.* genic SSRs and these markers used to amplify the mungbean genome are mostly other legume crop specific. The SSR markers from within *Vigna* species (cowpea, common bean, adzuki bean) and other genera such as soybean have been applied in mungbean and of these adzuki bean and common bean SSR markers showed a high rate of amplification of 72.7% and 60.6% [25,26]. The unigene based SSR markers showed a high transferability rate of 88% in different *Vigna* species [27].

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The SSR markers are most preferably used in the limited resourceful laboratories due to non-affordability of modern technologies [28]. The general methodology of SSR development consists of three steps *i.e.* preparation of SSR library, PCR and sequencing. This process is very cumbersome and expensive. Now a day's several workers [29,30,31,32,22,33,34,35,36,37] have developed SSR markersbutstill limited SSR markers are available in mungbean. This has further limited the molecular mapping of many desirable characters pertaining to stress resistance in the crop. Trait based mapping is urgently required for mungbeanto strengthen the molecular marker based improvement programme.With the help of next generation sequencing (NGS) technologies, it has become possible to develop and identify large numbers of SSR and other markers at low price. NGS technologies, coupled with bioinformatics approaches can massively increase the number of SSRs availability for carrying outgenetical investigations in under studied and economically important crops such as mungbean. Whole genome sequences of mungbean and urdbean varieties (ML 267 and Mash 114) was assembled at Punjab Agricultural University (PAU), Ludhiana and an aggregate of 443,867 SSR markers were discovered in *V. radiata* (cv. ML267) and *V. mungo* (cv. Mash 114), of which 4,10,282 were found polymorphic *in silico*. In present study, of the 250 WGS based SSR markers, a set of 200*in silico* polymorphic SSRs were validated for their transferability across different *Vigna* species and elucidating the underlying genetic diversity in genus *Vigna*.

## 2. Materials and Methods

### *2.1. Plant Material*

Phenotypically diverse accessions fromdiverse geographic regions were included to enhance the likelihood of detecting polymorphic marker loci. The diversity panel comprising of 25 *Vigna* accessions across 13 species were procured from ICAR-Indian Institute of Pulse Research (IIPR), Kanpur, Uttar Pradesh (Table 1). The present work was carried out at experimental area of Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana located at 244 meter above mean sea level (AMSL) (latitude: $30^0$ 90'N and longitudes: $75^0$ 85' E)with semi-arid climate zone.Each accession was sown in a single line in a bed of 3meter length at spacing of 40 cm between rows during *kharif* season, 2019.

**Table 1.** List of *Vigna* accessions genotyped in the study.

| Designation | Accessions | Designation | Accessions |
|---|---|---|---|
| GP1 | *V. umbellata*(Cultivated) | GP14 | *V. stipulacea* |
| GP2 | *V.umbellata*(Cultivated) | GP15 | *V. radiata*var.*radiata* |
| GP3 | *V. umbellata* | GP16 | *V. radiata*var.*mungo* |
| GP4 | *V. sublobata* | GP17 | *V. radiata*var.*mungo* |
| GP5 | *V. sublobata* | GP18 | *V. radiata*var.*mungo* |
| GP6 | *V. trilobata* | GP19 | *V. slyestris* |
| GP7 | *V. trilobata* | GP20 | *V. glabrescence* |
| GP8 | *V. trilobata* | GP21 | *V. radiata*var.*satulosa* |
| GP9 | *V. trilobata* | GP22 | *V. vexillata* |
| GP10 | *V. aconitifolia* | GP23 | *V. hainiana* |
| GP11 | *V. aconitifolia* | GP24 | *V. dalzelliana* |
| GP12 | *V. aconitifolia*(TMV-1) | GP25 | *V. unguiculata* |
| GP13 | *V. stipulacea* | | |

### *2.2. DNA Extraction and Quantification*

Total genomic DNA was isolated from fresh young and tender leaves of each accession employing standard CTAB method [38]. RNA contamination was removed with RNase at $37^0$C for 45 minutes. The quantity and quality of DNA was examined with agarose gel (0.8%) with lambda

 doi:10.20944/preprints202308.0658.v1



DNA as reference. The integrity and quantity of DNA based onagarose gel was optimized to 20 ng/μland used for amplification process.

*2.3. SSR Markerdesigning*

The whole genome contig assembly and scaffolding of WGS based SSR markers was done by using CLC assembler and SOAP de novo respectively at School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana and further used for SSR mining and identification of *in silico* polymorphism through MIcroSAtelllite (MISA). A total of 2,18,508 and 2,25,359 SSRs were detected from 4,71,725 and 4,44,059 sequences in *V. mungo* cv. Mash114 and *V. radiata* cv. ML267 respectively (Table 2) [39]. Using these SSR sequences, 250 *in silico* polymorphic mungbean SSR primers were mapped on urdbean contigs based upon 20-50 bp distance between mungbean and urdbean using e-PCR. The *in silico* polymorphism was discovered by e-PCR of SSR markers obtained from *V. radiata* cv. ML267 and *V. mungo* cv. Mash114. Descriptive information including strand, marker type, repeat numbers, contig, amplicon size, GC content, start- and end-position and chromosome numbers were obtained for each SSR markers. The primers were designed using Primer3 software with deafault parameters; melting temperature (55-65°C), guanine-cytosine (GC) content (40-70%), primer size of 18-27 bp length and product size of 150-280 bp. A series of250 di-nucleotide whole genome sequence (WGS) based SSR markers were synthesized from Promega Biotech and of these 200 SSR markers were used for validation of WGS derived polymorphism as well as transferability to other *Vigna* species (Table S1) (Figure 1).

**Table 2.** Summary of SSR Mining and frequency of different repeat types identified through whole genome sequencing of (*V. radiata* cv. ML267) and (*V.mungo* cv. Mash114) (Shivani, 2018).

| **Parameters** | **Number of SSR** | |
|---|---|---|
| | ***V. radiata* cv. ML267** | ***V. mungo* cv. Mash114** |
| **SSR Mining** | | |
| SSR sequences examined | 444,059 | 471,725 |
| SSRs identified | 225,359 | 218,508 |
| SSR containing sequences | 130,125 | 126,749 |
| Sequences containing more than 1 SSR | 50,760 | 46,626 |
| SSRs present in compound formation | 16,201 | 15,565 |
| **Repeat Type**[a] | | |
| Mononucleotide | 173,536 (77%) | 170,071 (77.83%) |
| Dinucleotide | 29,559 (13.12%) | 27,625 (12.64%) |
| Trinucleotide | 19,732 (8.76%) | 18,490 (8.46%) |
| Tetranucleotide | 1939 (0.86%) | 1794 (0.82%) |
| Pentanucleotide | 410 (0.18%) | 369 (0.17%) |
| Hexanucleotide | 183 (0.08%) | 159 (0.08%) |

[a] Data in parentheses is the percentage value of the repeat type.

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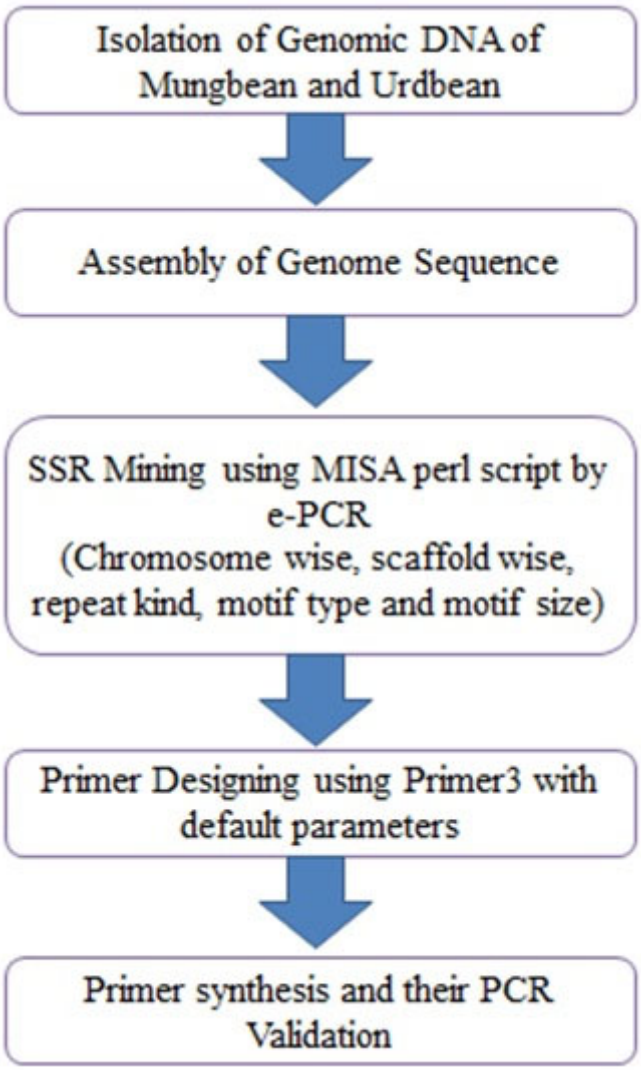


**Figure 1.** Primer mining, designing and PCR validation of WGS based SSR markers.

*2.4. SSR Validation*

The PCR reaction (SSR amplification) was performed in total of 20 µl reaction volume with 40 ng/µl of DNA template, 10µM of primer (forward and reverse), 10 mMdNTPs, 4.0 µl of 5X PCR buffer, 25mM of $MgCl_2$ and one unit of 5 µl Taq polymerase (Promega). The PCR profile for the amplification of DNA was set as denaturation at 94°C for 3 minutes, annealing at 55°C which comprised of 35 cycles and followed by extension at 72°C for 10 minuts. The amplified PCR product was run on 2.5% agarose gel, stained with ethidiumbromidein horizontal gel electrophoresis unit and visualized under gel documentation system (Alpha Imager, USA).

*2.5. GeneticDiversity, AMOVA and PCoA in Vigna Species*

Amongst the 25 accessions, the total number of alleles, amplicon size, and number were recorded in each of *Vigna* species. The amplified fragment were scored in base pair size and convert to 1 (amplification) and 0 (no amplification) format. Then each marker was assessed for number of alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), unbiased heterozygosity (uHe), Shannon Information Index (I) and fixation index (F) were calculated using GENALExV.6.5 software [40]. TheGENALExV.6.5 also used todetect population differentiation utilizing SSRmarkers byanalysis of molecular variance (AMOVA) and principal co-ordinate analysis (PCoA).

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*2.6. Population Structure Analysis*

Population structure analysis was carried out with STRUCTURE V.2.3.1 [41]. For identification of number of populations (K), the project run time was set to 100,000 Markov Chain Monte Carlo (MCMC) iterations and 100,000 burning period length with probability of admixture and independent allele frequency. The K value was setwith each10 independent run between 1 – 10 K. The optimal Delta-K value was determined with STRUCTURE HARVESTER [42]. Further, the accessions were assembled into clusters based upon the dissimilarity matrix using unweighted pair group method with arithmetic mean (UPGMA) neighbor joining method using DARwin6 software [43].

*2.7. Data Analysis*

2.7.1. Polymorphic Information Content (PIC)

Polymorphic information content (PIC) value provides an estimate of the discriminatory power of a locus or loci, by taking into consideration of number of alleles; relative frequency of alleles was estimated using the Botstein et al. [44] equation.

$$PIC = 1 - \sum_{i=1}^{n} (P_{ij})^2 - \{\sum_{i=1}^{n} (P_{ij})^2\}^2 + \sum_{i=1}^{n} (P_{ij})^2\}^2$$

where, Pij is the frequency of j$^{th}$ allele in the i$^{th}$ primer and summation extends over 'n' patterns.

2.7.2. Effective Multiplex Ratio (EMR)

The average number of DNA fragments amplified or detected per genotype using a marker system is considered as multiplex ratio (n). The number of loci polymorphic in the germplasm set of interest, analyzed per experiment is known as effective multiplex ratio.

$$Effective\ Multiplex\ Ratio\ (EMR) = n\ x\ \beta$$

where, n = Average number of fragments amplified by a genotype

β = Fraction of polymorphic band to the total polymorphic and monomorphic bands

$$\beta = \frac{PB}{(PB + MB)}$$

where,

PB = Number of polymorphic bands,

MB = Number of monomorphic bands

2.7.3. Marker Index (MI)

It is measured as product of polymorphic information content (PIC) and effective multiplex ratio (EMR). It is estimated using formula given by Powell et al. [45]

$$Marker\ Index\ (MI) = PIC\ \ x\ EMR$$

where,

PIC = Polymorphic information content

EMR = Effective multiplex ratio

2.7.4. Resolving Power (RP)

It is the measure of ability of each primer to detect level of variation between individuals. It is calculated according to Prevost and Wilkinson [46].

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$$Resolving\ Power\ (RP) = \sum Ib$$

where, Ib = Informative fragments

$$Ib = 1 - [2(0.5 - Pi)]$$

where, Pi = Proportion of genotypes containing the $i^{th}$ band

## 3. Results

### *3.1. WGS Based SSR Markers Development*

The whole genome sequencing (WGS) of ML267 and Mash114 was performed by [39] at School of Agricultural Biotechnology, PAU, Ludhiana. From the WGS, a total of 4,43867 SSRs were identified in *V. radiata* cv. ML267 and *V. mungo* cv. Mash114, of which 4,10,282 poly SSR primers were designed *in silico* by e-PCR. Out of these primers, a total of 250 in silico polymorphic mungbean SSR primers were mapped on urdbean contigs based upon the 20-50 bp distance between mungbean and urdbean using e-PCR (Figure 1). These 250 SSR markers were flanking dinucleotide SSR motifs and covered all 11 linkage groups of mungbean and urdbean. A maximum of 45 SSRs were from chromosome 7 and a minimum of 8 SSRs from chromosome 9 were used. The remaining SSR (197) were distributed unevenly as 41, 36, 24, 21, 18, 17, 14, 13 and 13 on chromosome 5, 8, 6, 1, 11, 4, 3, 2 and 10. Among these SSR repeats, 10 different dinucleotide repeats: (AT)n, (AG)n, (AC)n, (TA)n, (TG)n, (TC)n, (GA)n, (GT)n, (CA)n and (CT)n were observed (Table 3). The number of (AT)nand (TA)n repeats were most abundant dinucleotide repeat motifs 69 (27.6%) and 62 (24.8%) respectively. These two dinucleotide repeats (AT/TA) accounting 52.4% of the total repeat motifs.

**Table 3.** Abundance of dinucleotide repeats in *in-silico* developed SSR markers between *V. radiata* (cv. ML267) and *V.mungo* (cv. Mash 114).

| Dinucleotide repeat | n | Number | Percentage |
|---|---|---|---|
| (AT)n | 6, 7, 8, 9, 10, 11, 12, 13, 14, 17 | 69 | 27.6 |
| (AG)n | 6, 7, 8, 9, 10, 13, 14, 16, 17, 22 | 30 | 12.0 |
| (AC)n | 6, 7, 8, 9 | 16 | 6.40 |
| (TA)n | 6, 7, 8, 9, 10, 11, 12, 13, 20 | 62 | 24.8 |
| (TC)n | 6, 7. 8, 9, 10, 11, 13, 14, 16, 17 | 31 | 12.4 |
| (TG)n | 6, 7, 9 | 11 | 4.40 |
| (CT)n | 6, 7, 8, 9, 12, 14 | 13 | 5.20 |
| (CA)n | 6,7 | 02 | 0.80 |
| (GA)n | 6, 7, 8, 10, 12. 19, 20 | 10 | 4.00 |
| (GT)n | 6, 7, 14 | 06 | 2.40 |

### *3.2. Validation of SSR Markers on Vigna Species Accession for Transferability Studies*

For validation, a set of 25 different *Vigna* species accessions belonging to 13 species were genotyped with 200 WGS developed SSR markers. All of these 200 SSR markersproduced varying level of amplification in all the accessions, except four [one of *V. radiata* var. *radiata* (GP15) and three of *V. mungo* var. *mungo*(GP16, GP17 and GP18)] (Table S2). Out of these 200 SSRs used for validation, 130 markers (65%) showed polymorphismwhile 70 markers (35%) exhibited monomorphism in the different *Vigna* species accessions.

Size-based polymorphism was observed by 402 alleles of the total amplified 2121 alleles with an average of 8.1 alleles per locus.The PCR amplification profile of WGS based SSR markers in different *Vigna* accessions is given in Figure 2.The number of alleles amplified by WGS-SSRs were ranged as 7 (SSR 274) to 24 (SSR 271). The average number of alleles amplified per marker was estimated at 15.7. Seven SSR markers *viz.*, SSR 271 (24 alleles), SSR 123 (23 alleles), SSR 208, SSR 262, SSR 273, SSR 287

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and SSR 289 (21 alleles) amplified more than 20 alleles. With respect to the *Vigna* species accessions, the minimum of 8 accessions and maximum of 21 accessions showed PCR amplification with these WGS derived SSR markers and the amplicon size varied from 50-1000 base pairs (Table S2).

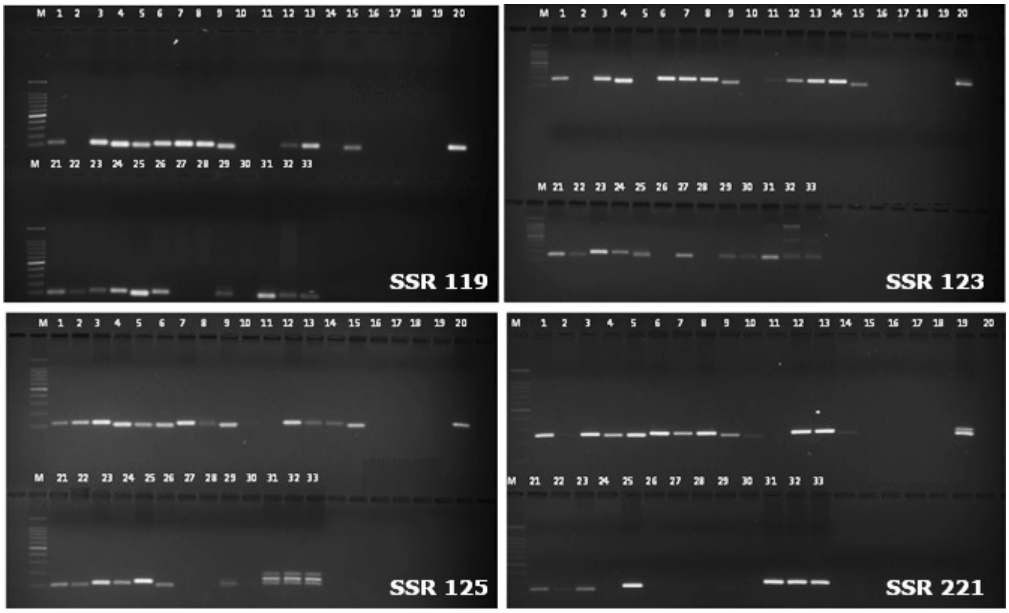


**Figure 2.** Transferability of WGS based SSR markers in different *Vigna* accessions.(1) *V.umbellata*(Cultivated); (2) *V.umbellata*(Cultivated); (3) *V. umbellata;*(4) *V.sublobata;*(5) *V.sublobata;*(6) *V.trilobata;*(7) *V.trilobata;*(8) *V.trilobata;*(9) *V.trilobata;* (10) *V.aconitifolia;*(11) *V. aconitifolia;*(12) *V.aconitifolia*(TMV-1); (13) *V. stipulacea;*(14) *V. stipulacea;*(15) *V. radiate* var. *radiata;*(16) *V. radiata*var.*mungo;*(17) *V. radiata*var.*mungo;*(18) *V. radiata*var.*mungo;*(19) *V. slyestris;*(20) *V. glabrescence;*(21) *V. radiata*var.*satulosa;*(22) *V. vexillata;*(23) *V. hainiana;*(24) *V. dalzelliana;*(25) *V. unguiculata.*

### *3.3. SSR Marker Analysis*

The marker analysis based upon the average PIC estimates for all the markers arrayed between 0.09 (SSR 262) to0.84 (SSR 269)with 0.31 as an average PIC value (Table S3). Of the 130 polymorphic markers, 85 markers (65.38%) were highly informative (PIC ≥ 0.45), 26 (20.50 %) reasonably informative (PIC = 0.25 – 0.45) and 19 (14.62 %) as slightly informative (PIC < 0.25). The MI value ranged between 0.091 (SSR 262) to 3.342 (SSR 269). Similarly, the EMR also varied from 1.0 to 4.0 (SSR 269). The average MI and EMR for the 130 polymorphic markers was recorded as 0.54 and 1.01 respectively. The RP for all 200 SSR markers varied from 0.56 (SSR 274) to 2.00 (SSR 177) with an average value of 1.27 (Table S3). The other marker utility parameters such as observed and effective allele number, Shannon diversity index and estimates of heterozygosity were also computed (Table S3). The effective number of alleles (Ne) ranged between one to two(average estimate 1.374) and the Shannon diversity index (I) varied as 0.693 to 0(average estimate 0.321). The Shannon information index was the highest for SSR106 and SSR 234 (0.693), followed by SSR253 (0.686), SSR 198 and SSR 251 (0.685), SSR 156 (0.683), SSR241 (0.679), SSR 135 (0.675). The value for observed heterozygosity was obtainedfrom0.188 to 0with 0.016 averageswhile the estimate of expected heterozygosityordered from 0.50 to 0with 0.216 average values. The unbiased expected heterozygosity (uHe) was recorded between 0to 0.526 with an average of 0.235.

### *3.4. Genetic Diversity Andrelationship Among the Different Vigna Species*

The *Vigna* accessions were clustered into three main clusters based upon genetic dissimilarity estimated using unweighted pair group method with arithmetic mean (UPGMA) neighbor joining approach (Figure 3). Cluster 1 consists of 10 *Vigna* accessions which were further divided into two major sub-clusters (Sub-cluster 1a and sub-cluster 1b). Sub-cluster 1a included seven accessions {GP5

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(*V. sublobata*), GP4 (*V. sublobata*), GP9 (*V. trilobata*), GP15 (*V. radiata* var. *radiata*), GP22 (*V. vexillata*), GP21 (*V. radiata* var. *setulosa*) and GP20 (*V. glabrescence*)} while, sub-cluster 1b comprised of three accessions {GP 24 (*V. dalzelliana*), GP23 (*V. hainiana*) and GP25 (*V. unguiculata*)}. The second cluster comprised of nine accessions with 8 {GP17 (*V. radiata* var. *mungo*), GP16 (*V. radiata* var. *mungo*), GP18 (*V. radiata* var. *mungo*), GP19 (*V. slyestris*), GP11 (*V. aconitifolia*), GP10 (*V. aconitifolia*), GP2 (*V. umbellata* cultivated) and GP1 (*V. umbellata* cultivated)} and 1 accessions (GP12) in sub-cluster 2a and 2b, respectively. The third cluster consisted of 6 accessions that grouped into two sub-clusters namely 3a having 5 accessions {GP8 (*V. trilobata*), GP6 (*V. trilobata*), GP7 (*V. trilobata*), GP14 (*V. stipulacea*) and GP13 (*V. stipulacea*)}, and 3b with one accession (GP3 *V. umbellata*).Nei's unbiased genetic distance (GD) and genetic identity (GI) were also estimated and based upon the genetic distance, the *Vigna* accessions were categorized into four populations (pops) (Table 4). The genetic distance between pops ranged from 0.189 (between Pop 4 and 3) and 0.458 (between Pop 4 and 2). The *Vigna* accessions of Pop 4 and 3 are closely related while accessions from Pop 4 and 2 are distantly related. Pop 1 and Pop3 comprised, four (one of *V. sublobata* and three of *V. mungo*) and three accessions (one of *V. sublobata*, one *V. radiata* var. *radiata* and one of *V. radiata* var. *setulosa*). While pop 2 comprised of highest of 11 *Vigna* accessions (three accessions each of *V. umbellata, V. trilobata*; two each of *V. aconitifolia, V. stipulacea* and one accession of *V. glabrescence*) followed by pop 3 having seven accessions (one accession each of *V. unguiculata, V. trilobata, V. aconitifolia var.* TMV, *V. sylvestris, V. vaxillata, V. hainiana* and *V. dalzelliana*).

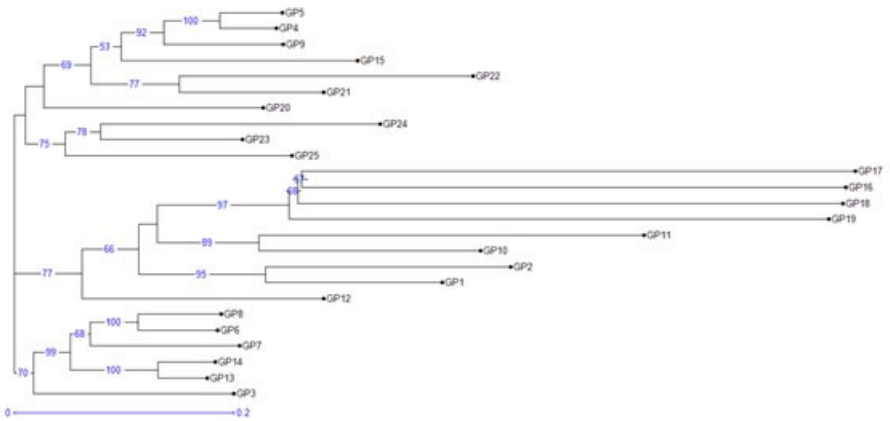


**Figure 3.** Cluster analysis of different *Vigna* accessions based on neighbor-joining method.

**Table 4.** Nei's unbiased measures of genetic identity and genetic distance based upon 200 SSR markers.

| Pop ID | Pop 1 | Pop 2 | Pop 3 |
|---|---|---|---|
| **Pop 2** | 0.374 | - | - |
| **Pop 3** | 0.208 | 0.442 | - |
| **Pop 4** | 0.250 | 0.458 | 0.189 |

*3.5. Population Structure Analysis*

The population structure analysis of 25 *Vigna* accessions was performed with 130 polymorphicSSR markers. Based upon the admixture model with independent alleles, the maximum delta K value (144.79) draws a sharp peak at K=2 (Figure 4) which divided genotypes into two sub-populations (SP1 and SP2)} (Figure 3). The SP1 comprised of 11 accessions whereas, SP2 had 14 accessions. The sub-population 1 (SP1) included accessions GP1 (*V. umbellata* cultivated), GP2 (*V. umbellata* cultivated), GP10 (*V. aconitifolia*), GP11 (*V. aconitifolia*), GP12 (*V. aconitifolia* TMV-1), GP16 (*V. radiata* var. *mungo*), GP17 (*V. radiata* var. *mungo*), GP18 (*V. radiata* var. *mungo*), GP22 (*V. vexillata*) and GP24 (*V. dalzelliana*) while, SP2 included GP3 (*V. umbellata*), GP4 (*V. sublobata*), GP5 (*V. sublobata*),

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GP6 (*V. trilobata*), GP7 (*V. trilobata*), GP8 (*V. trilobata*), GP9 (*V. trilobata*), GP13 (*V. stipulacea*), GP14 (*V. stipulacea*), GP15 (*V. radiata* var. *radiata*), GP19 (*V. slyestris*), GP20 (*V. glabrescence*), GP21 (*V. radiata* var. *setulosa*), GP23 (*V. hainiana*) and GP25 (*V. unguiculata*).

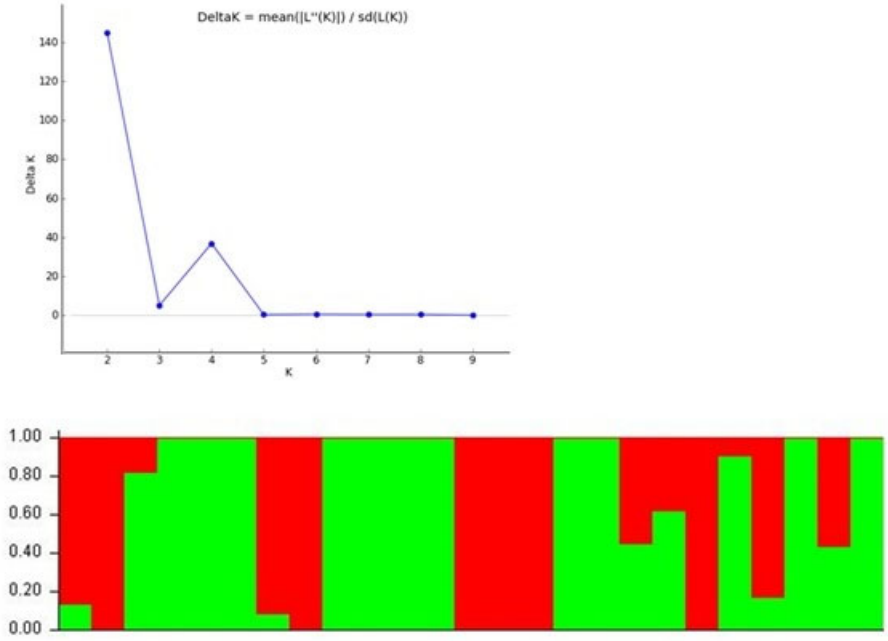


**Figure 4.** Population structure analysis of 25 *Vigna*accessions based on 200 SSR markers (K=2) and graph of estimated membership fraction for K=3.

*3.6. Analysis of Molecular Variance (AMOVA) and Principal Co-Ordinate Analysis (PCoA)*

Analysis of molecular variance was performed within and among individuals diversity module. Significant higher genetic variance was observed among the individuals (89%) as compare to within individuals (1%) (Table 5 and Figure 5). Principal co-ordinate analysis (PCoA) revealed that the first and second integral coalitiondeciphering 50.79 and 15.42 per cent of total variance. The PCoA categorized the accessions into four groups involving different species similar to UPGMA-neighbour joining clustering (Figure 6). The biplot PCA showed correlation with UPGMA based phylogenetic tree with respect to grouping of *Vigna* species accessions.

**Table 5.** Summary of Analysis of Molecular Variance (AMOVA).

| Source of Variation | Df | Sum of square | Mean Sum of square | Estimated Variance | % Variance | F statistics |
|---|---|---|---|---|---|---|
| Among population | 2 | 470.62 | 235.31 | 7.24 | 10 | 0.105 |
| Among individual | 22 | 2706.18 | 123.00 | 61.15 | 89 | 0.989 |
| Within individual | 25 | 17.50 | 0.700 | 0.70 | 1 | 0.990 |
| Total | 49 | 3194.30 | | 69.09 | 100 | |

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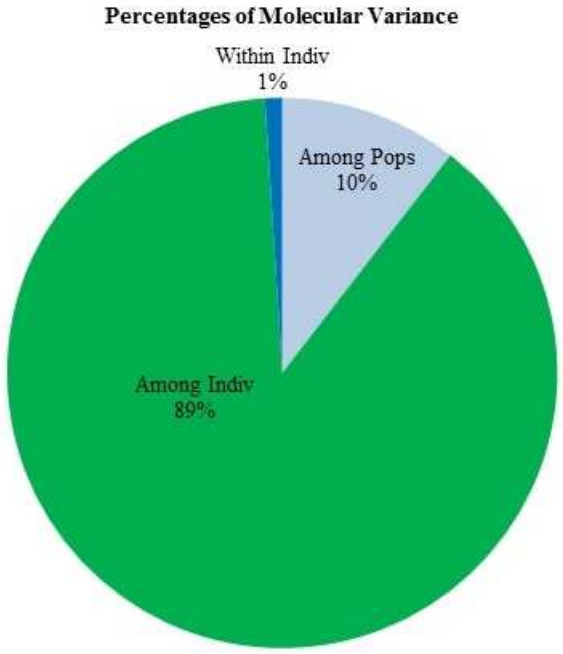


**Figure 5.** Analysis of Molecular variance (AMOVA) showing the percentage of molecular variance among and within populations and among the various *Vigna* species genotypes.

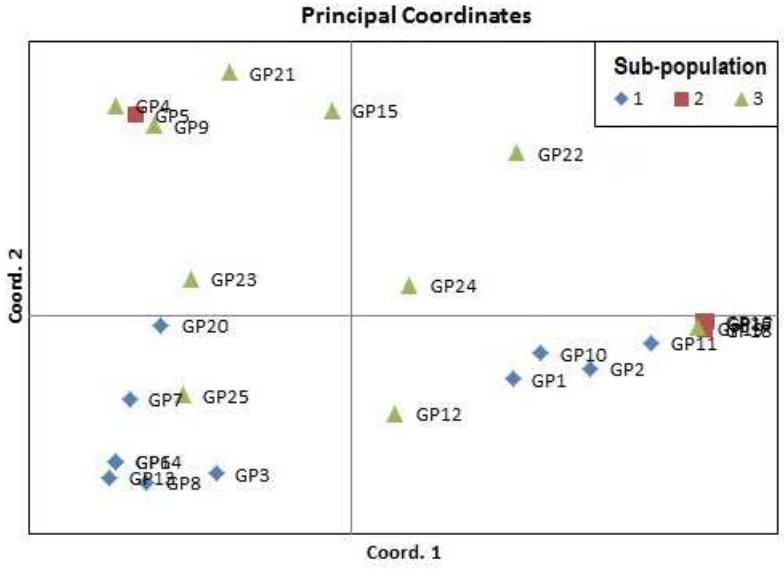


**Figure 6.** Principal co-ordinate analysis (PCA) showing the distribution of *Vigna* species accessions.

## 4. Discussion

Crop improvement is important for every crop species to make it available for mankind. For every crop improvement programmes, availability of accessible genetic variation in the crop genetic resources is indispensable. Determination of genetic diversity provides opportunity for exploitation of useful variation present in the available germplasm in breeding programme as promising parents [47]. Pre-breeding is an approach that harnesses the useful variability in unadapted genetic material which cannot be utilized as such in breeding populationsand serves necessarily as the major stride for employing utilization of genetic variation in improvement programmes [48,49,50,51,52,53,54,55]. The genetic variability existing in the gene banks helps in conservation, characterization and implementation of genetic variation in crop improvement programmes [56]. The *Vigna* gene pool

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serves as source of ample amount of untapped genetic polymorphism is available in the wild *Vigna* species [6,57,4]. For unlocking the available genetic variation, DNA based molecular markers are required but limited genomic resources is available in mungbean.

The present study involved validation of 200 SSR markers out of 250 which were developed from mungbean cv. ML 267 and urdbean cv. Mash 114 using whole genome sequence strategy at School of Agriculture Biotechnology (SAB), Punjab Agricultural University (PAU), Ludhiana [39]. These SSR markers were flanking dinucleotide SSR motifs and covered all 11 linkage groups of mungbean and urdbean. Chromosome 7 has the maximum number of 45 SSR markers whereas chromosome 9 has minimum of 8 SSR markers. Rest of the SSR markers are distributed unevenly on 5, 8, 6, 1, 11, 4, 3, 2 and 10 chromosomes. The SSR markers are comprised of ten different types of dinucleotide repeat motifs and two repeat motifs *i.e.*(AT)n and (TA)n were predominant. These two dinucleotide repeats (AT/TA) accounted 52.40 per cent of total repeat motifs. In general, it has been observed that di-nucleotide repeats are mainly present in many legume crops [58] but tri-nucleotide repeats have been commonly found in mungbean [35,59,60] and in other legume crops as in pea [61], cowpea [27], chickpea [62], common bean [63] and horse gram [64]. The mononucleotide repeat motifs have been observed in relative abundance in mungbean [65]. Higher number of mono- and tetra-nucleotide repeats was also reported from transcriptome sequencing of adzuki bean [66]. The transcriptome based SSRs can be developed from mononucleotide repeat because such type of markers exhibit high polymorphism. Similarly, the whole genome based SSR developed from mononucleotide repeats will also be more polymorphic than other repeats. However, the chances of error like DNA slippage during PCR amplification by polymerase enzyme machinery cannot be ruled out. Hence, in order to overcome this limitation, dinucleotide repeats were selected for the study.

Simple sequence repeats (SSR) are tandem repeated sequences (1–6 nucleotides), having high rate of polymorphism, reproducibility, co-dominant nature and abundantly distributed throughout the genome. SSRs exhibited excellent degree of transferability betwixt and amongst the closely related species or genera which makes SSR useful molecular marker for the estimation of variation at gene level, mapping of economically important loci and breeding programmes based on molecular markers. The SSR marker transferability is relied on the divergence betwixt the individual accessions. The closer the genetic distance betwixt the accessions, higher the transferability of SSR markers [67]. Within the same species of same genus or across the related genus within families, SSR transferability is higherthan between different genus and families [68]. Marker transferability is a parameter to describe closeness and crossability between the species. Mungbean and other species specific SSR markers have been used in different studies for assessing polymorphism among and between the *Vigna* accessions or introgression lines. In accordance to the previous reports by Somta et al. [69]; Tangphatsornruang et al. [33]; Gupta et al. [70]; Dikshit et al. [2]; Singh et al. [71]; Gupta et al. [58]; SatinderKaur et al. [72]; Simranjit Kaur et al. [73], the present investigation showed more than 90 per cent marker transferability across the different *Vigna* accessions. The successful applicability of whole genome sequence based SSRs betwixt different *Vigna* species accessions showed that the flanking regions of these SSRs are adequately conserved amplification of genomic regions. The very high cross-species transferability percentage depends on the number of species analyzed and genetic distance among them.

The newly developed SSR markers in our study amplified 7 to 24 alleles (average estimate 15.7). The amplification of higher number of alleles is an indicative of the prevalent exalted genetic diversity among the *Vigna* species. The 4 to 16 alleles per locus have been obtained in the Asiatic mungbean accessions using 53 SSR markers [74]. In another study,GeetaKumari [75] reported 9 to 31 alleles per locus in 119 mungbean accessions of 19 *Vigna* species. Studies by Dachapak et al. [76], Sarr et al. [77] and Singh et al. [78] also amplified alleles in the range of 15-25 in zombie pea, cowpea and mungbean respectively. Heterozygosity and PIC value are the two important estimates of genetic diversity at genotypic level. The high PIC value in present study is in accordance with other studies [57,76,78,74,77,75] indicated that the microsatellite flanking regions are conserved and highly useful in inferring the phylogenetic relationship between a number of species. Higher estimates of MI and EMR of SSRs suggested high polymorphism of SSR markers. High resolving power (RP) of SSRs (0.5

 doi:10.20944/preprints202308.0658.v1



to 2.0) is another diversity parameter which revealed the marker power for distinguishing betwixt genotypes.Thus, it become clear that, SSR markers have potential in different genetic studies such as crop germplasm characterization, genetic diversity assessment; marker-trait association and marker assisted breeding which helps in development of improved versions of crop varieties.

In general, the results from PCoA and UPGMA clustering were not completely consistent with structure analysis. The progenitor species of mungbean and urdbean*i.e. Vignasublobata* and *Vignasilvestris* clustered in two separate clusters as they have been categorized under primary and secondary gene pool. While GeetaKumari et al. [75] reported the grouping of progenitor species in one sub-cluster. Mixed grouping of the members of all three gene pools were also observed after clustering. The primary gene pool (*V. radiata* var. *setulosa*) grouped with secondary (*V. trilobata*) and tertiary gene pool (*V. glabrescence, V. vexillata*) members under sub-cluster 1a. Similarly, secondary and tertiary gene pool species clustered together in one cluster with two sub-cluster. Similar observations have been recorded by GeetaKumari et al. [75] where secondary (*V. trilobata*) and tertiary gene pool (*V. dalzelliana, V. umbellata* and *V. vexillata*) species accessions clubbed in a sub-cluster.

Population structure analysis depicted two types of populations SP1 and SP2. The highest number of genotypes (14) was grouped into SP2. The accessions in SP1 were mainly of cultivated type whereas most of the wild relatives were grouped into SP2. Based upon the suitable K value which capture the best structure of population, Chen et al. [60] and Noble et al. [78] also divided the mungbean genotypes in cultivated and wild mungbean genotypes having higher genetic similarity. In other *Vigna*species like cowpea, the appropriate K value proved helpful in differentiating the genotypes based upon the geographical as well as genetic similarity [79,80,81]. The accessions of progenitors of mungbean and urdbean (*V. sublobata* and *V. silvestris*) and their relative species *V. radiata* var. *setulosa* and *V. radiata* var. *mungo* categorized separately in SP1 and SP2 while GeetaKumari et al. [75], Singh et al. [78], Pratap et al. [74], Sexena et al. [82], Pandiyan et al. [83], Kumar et al. [84] progenitors categorized with mungbean and urdbean accessions in one group. The secondary gene pool species accession of *V. aconitifolia* grouped with tertiary gene pool accessions of *V. umbellata, V. vexillata, V. dalzelliana*in SP1 due to their close relationship with each other [6]. Similar to GeetaKumari et al. [75] the *V. umbellata* and *V. trilobata* categorized into two groups (SP1 and SP2).The *V. hainiana, V. stipulacea, V. glabrescence* and *V. unguiculata* accessions categorized in SP2 as admixture.

AMOVA provides the clues regarding the genetic variation present within and among the individual. The greater variance of 89% among the individuals revealed the presence of high genetic diversity. The low genetic diversity among the population indicates the exchange of germplasm between different regions, distribution of similar *Vigna* species [60,80,75]. Among the population low level of genetic diversity of 10% has been observed our study while GeetaKumari et al. [75] obtained the high level of genetic diversity of 88.33% among the population. Fst is an estimate of population differentiation on account of genetic composition. Frankham et al. [85] stated that the Fst estimate <0.15 is an important criterion for population discrimination. The obtained Fst value of 0.105 is near to the significant value indicating the low differentiation between individuals. The results of principal co-ordinate analysis (PCoA) and UPGMA based clustering were in agreement showing gene diversity and clear differentiation of cultivated and wild *Vigna* species.

## 5. Conclusion

*Vigna* species gene pool harbors huge genetic diversity with variable alleles that can be harnessed for developing cultivars having high yield potential. Elucidating the underlying genetic variation present in both the wild and cultivated species will be helpful in widening the genetic base of breeding lines and marker assisted introgression of desirable traits into modern cultivars for successful genetic improvement programmes. In the present study the newly developed WGS based SSR markers are highly polymorphic in nature and showed high rate of cross-species transferability among *Vigna*accessions, which indicates their usefulness in pre-breeding and genetic

dissection of novel genes/QTLs linked with agronomic performance, nutritional quality, resistance to diseases and insect-pests and tolerance towards abiotic stresses.

**Authors' contributions:** PS, TSB and AS: Conceived and designed the study. PS: field and laboratory performed experiments. ISY: designed the SSR primers. NL and SAHP: assisted in designing SSR primers. KSM: assisted in field and laboratory experiments. JA: Analysis of data and proof-reading. PS: preparation of manuscript. PS, PS and SN: correction in manuscript. PS, TSB and AS: revised and edited the manuscript.

**Declaration of Competing Interest:** Authors of the manuscript stated that there is no competing interest regarding the financial as well as individual conflict.

**Acknowledgments:** Department of Biotechnology, Govt. of India, New Delhi is highly appreciative for providing funding and thankful to Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana for facilitating in conducting this research.

**Funding:**

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