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Genetic differentiation of *Vigna* species by RAPD, URP and SSR markers

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Abstract

Seventy genotypes belonging to 7 wild and cultivated *Vigna* species were genetically differentiated using randomly amplified polymorphic DNA (RAPD), universal rice primer (URP) and simple sequence repeat (SSR) markers. We identified RAPD marker, OPG13 which produced a species-specific fingerprint profile. This primer characterized all the *Vigna* species uniquely suggesting an insight for their co-evolution, domestication and interspecific relationship. The cluster analysis of combined data set of all the markers resulted in five major groups. Most of the genotypes belonging to cultivated species formed a specific group whereas all the wild species formed a separate cluster using unweighted paired group method with arithmetic averages and principle component analysis. The Mantel matrix correspondence test resulted in a high matrix correlation with best fit (r = 0.95) from combined marker data. Comparison of three-marker systems showed that SSR marker was more efficient in detecting genetic variability among all the *Vigna* species. The narrow genetic base of the *V. radiata* cultivars obtained in the present study emphasized that large germplasm collection should be used in *Vigna* improvement programme.

Additional key words: fingerprinting, green gram, molecular analysis.

Introduction

The genus *Vigna* composed of more than 200 species that are of considerable economic importance in many developing countries. Mung bean [*V. radiata* (L.) Wilczek], urd bean [*V. mungo* (L.) Hepper] and cowpea [*V. unguiculata* (L.) Walp.], are key dietary staples for millions of people. Additionally, adzuki beans [*V. angularis* (Willd.) Ohwi & Ohashi], bambara groundnuts [*V. subterranea* (L.) Verdn.], mat bean [*V. aconitifolia* (Jacq.) Marechal], and rice bean [*V. umbellata* (Thunb.) Ohwi & Ohashi] are also consumed in many countries. The pedigree and molecular analysis of commercial cultivars have revealed their narrow genetic base resulting in inherently low yield potential and susceptibility to biotic stresses like mungbean yellow mosaic virus (MYMV), powdery mildew (*Erysiphe polygoni*), cercospora leaf spot (*Cercospora canescens* Ell. and *C. cruenta* Sacc.), bruchids (*Callosobruchus maculates* F. and *C. chinensis* F.) and abiotic stresses like preharvest sprouting and drought. It is therefore, imperative to broaden the genetic base of cultivated *Vigna* species. Hence, analysis of the extent of genetic diversity present in the wild and cultivated *Vigna* germplasm is essential for their inclusion in future breeding programmes. Conventionally the genetic diversity has been estimated on the basis of the morphological markers. The availability of low number of morphological markers, their poorly known genetic control and environmental influence on phenotypic expression at different stages of growth are some of the established impediments in using these as

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Abbreviations: AFLP - amplified fragment length polymorphism; CTAB - cetyltrimethyl ammonium bromide; EMR - effective multiplex ratio; H_{av} - arithmetic mean heterozygosity; Hn - expected heterozygosity; ISSR - inter simple sequence repeats; MI - marker index; PCA - principal components analysis; PCR - polymerase chain reaction; RAPD - randomly amplified polymorphic DNA; Rp - resolving power; SAHN - sequential agglomerative hierarical nested; SAMPL - selective amplification of microsatellite polymorphic loci; SSR - simple sequence repeat; STMS - sequence tagged microsatellite; UPGMA - unweighted paired group method with arithmetic averages; URP - universal rice primer.
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stable genetic markers in the diversity analysis. Molecular analysis of genetic diversity in *Vigna* species have been performed by using DNA markers like randomly apmlified polymorphic DNA (RAPD; Samec *et al.* 1998, Santalla *et al.* 1998, Banerjee *et al.* 1999, Lakhanpaul *et al.* 2000, Lambridges *et al.* 2000, Betal 2004), inter simple sequence repeats (ISSR; Souframanien *et al.* 2004), restriction fragment length polymorphism (RFLP; Lambridges *et al.* 2000), amplified fragment length polymorphism (AFLP; Zong *et al.* 2003) and simple sequence repeat (SSR; Wang *et al.* 2004) markers. Similarly, Tosti and Negri (2002) studied the efficiency of three PCR based markers RAPD, AFLP, and selective amplification of microsatellite polymorphic loci (SAMPL) in detecting genetic variation in cowpea (*Vigna unguiculata*) landraces. However, there are limited reports on the extensive molecular analysis of *Vigna* species and comparative analysis of different marker systems because of unavailability of more robust PCR based markers like sequence tagged microsatellite (STMS) and SSR markers. An attempt has therefore been made in the present study, to combine RAPD, universal rice primers (URP) and SSR markers derived from other plant species for the genetic analysis of *Vigna* species. The objectives of present investigation were, to characterize genetic variability in different accessions and cultivars of *Vigna* and to establish the utility of different marker systems.

Materials and methods

Plants: Seventy genotypes of various indigenous and exotic collections belonging to seven species of *Vigna* were used in molecular analysis (Table 1). These included *Vigna radiata* var. *sublobata* (wild, 4 accessions), *Vigna mungo silvestris* (wild, 8 accessions), *Vigna radiata setulosa* (wild, 1 accession), *Vigna umbellata* (cultivated, 4 accessions), *Vigna unguiculata* (cultivated, 10 accessions). *Vigna mungo* (cultivated, 7 accessions) and *Vigna radiata* (cultivated, 36 accessions).

Genomic DNA isolation and purification: The genomic DNA was isolated from 5-d-old seedlings by using cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). Young leaf tissue (5 g) was grounded in liquid nitrogen by using pre-chilled motor and pestle. Leaf tissue powder was transferred to polypropylene tube containing 25 cm^3 DNA extraction buffer (50 mM Tris HCl, 150 mM NaCl, 100 mM EDTA, 10 % CTAB and 0.002 cm^3 of β-mercaptoethanol). The tubes were incubated at 65º C for 30 min with occasional gentle swirling. Two third volume of chloroform: isoamyl alcohol (24:1, v/v) was added to this sample and mixed. The sample was kept at -20 ºC for 15 - 25 min and then centrifuged at 10 000 *g* for 10 min at 4 ºC in *Sorvall* (USA) centrifuge (rotor *RC 5B Plus*). DNA pellet was rinsed with 70 % ethanol for 10 - 15 min. This pellet was dried at room temperature overnight. The dried pellet was dissolved in TE buffer (pH 8.0). For purification of DNA, RNAse treatment was given as described by Murray and Thompson (1980). The purified DNA was quantified by running 0.002 cm^3 of each DNA sample on 0.8 % agarose gel along with uncut lambda DNA (30 and 60 ng) to adjust final concentration of 10 - 12.5 ng mm^{-3} for use in PCR analysis.

Table 1. Sources of accessions of different *Vigna* species used in molecular analysis. NBPGR - National Bureau of Plant Genetic Resources, New Delhi, India, IARI - Indian Agricultural Research Institute, New Delhi, India, IIPR - Indian Institute of Pulse Research, Kanpur, India, AVRDC - Asian Vegetable Research Development Center, Thailand, IC - indigenous collection, EC - exotic collection

Species	Serial number, name of accession/cultivar	Source
Vigna radiata var. *sublobata*	1. IC253671 2. IC331442 3. IC203647 4. IC 253924	NBPGR
Vigna mungo var. *silvestris*	5. IC203709 6. IC203670 7. IC203704 8. IC203717 9. IC203722 10. IC253919 11. IC263674 12. IC253919	NBPGR
Vigna radiata var. *setulosa*	13. IC251419	NBPGR
Vigna umbellata	14. VUM241 15. VUM66 16. RBL-1 17. MNPL3	NBPGR
Vigna unguiculata	18. EC458401 19. V130 20. EC170584-13 21. EC170584-12 22. C152 23. V585 24. DCP2 25. GC3 26. IC259091 27. V578	IARI, AVRDC
Vigna mungo	28. SIC 29. PU19 30. PSIC4 31. PSIC3 32. PS2C 33. PU35 34. PSC1	NBPGR
Vigna radiata	35. IPM03-101 36. IPM03-102 37. IPM03-103 38. IPM-104 39. IPM-105 40. IPM-106 41. IPM-107 42. IPM-108 43. IPM-109	IIPR
	44. IPM-110 45. IPM-111 46. IPM-112 47. IPM-113 48. IPM-114 49. IPM-115 50. PLM334 51. M1319(B) 52. IC11303-3 53. PLM250	NBPGR, AVRDC
	54. EC398844 55. IC8986-B-2 56. PLM1097 57. IC114 58. PLM829 59. EC398884 60. PLM777 61. PLM975 62. TM96-2 63. PLM625 64. PDM139 65. NM-1shining 66. IPM02-1 67. IC8961-5 68.V.S.N.	IIPR
	69. EC30400 70. EC398897	NBPGR, AVRDC

Primer selection and polymerase chain reaction: Forty RAPD primers (from Kits OPC, OPE, OPF and OPG), were obtained from *Operon Technologies Inc*., Almeda, CA, USA. Five universal rice primers (URPs) originally derived from the repeat elements of weedy rice by Kang *et al*. (2002) were got synthesized from the *Operon Technologies*. One SSR primer designed from the *ferritin* gene (Acc.No.X58274) was derived from *Phaseolus vulgaris* (Yu *et al*. 2000). A total of 14 primers (8 RAPDs, 5 URPs and one SSR) were selected for the analysis of all the species as other primers produced indistinct amplification products. The list of primers, their sequences and annealing temperature along with some of the characteristics of the amplification products obtained in different *Vigna* species is given in Table 2.

For RAPD, URP and SSR analysis, PCR amplifications were carried out in a total volume of 0.025 cm^3 containing 25 - 30 ng of genomic DNA (Williams *et al*. 1990). The PCR reaction mixture consisted of 10× PCR buffer (0.01 % gelatine, 20 mM Tris-HCl, pH 8.4; 50 mM KCl, 1.5 mM $MgCl_2$), 10 mM dNTPs, 0.2 U *Taq* DNA polymerase, and 0.2 µM primer. All the PCR reaction components were obtained from *Bangalore Genei Pvt. Ltd*., Bangalore**,** India. The PCR amplification was performed by using thermal cycler (*T-Gradient Biometra*, *GmBh*, Göttingen, Germany). For RAPD, PCR temperature profiles were as follows: initial DNA denaturation at 94 ºC for 5 min, followed by 40 PCR cycles at 94 ºC for 1 min, 35 ºC for 1 min, 72 ºC for 2 min and a final cycle at 72 ºC for 7 min. For both URP and SSR markers, PCR reactions were performed with initial DNA denaturation at 94 ºC for 5 min followed by 35 cycles at 94 ºC (1 min) for DNA denaturation, 45 ºC and 42 ºC (1 min) for primer annealing for URP and SSR markers, respectively, 72 ºC (2 min) for primer extension and final extension at 72 ºC for 7 min. All the amplified PCR products obtained from RAPD and URP markers were resolved by electrophoresis on 1.4 % agarose gel and SSR marker at 3.5 % agarose gel for 3 h in 1× TBE buffer, stained with ethidium bromide and the photographs were taken by using *Gel Documentation System* (*Alpha Innotech Corporation*, USA).

Data analysis: DNA bands obtained with RAPD, URP and SSR markers were scored visually for the presence (1) and absence (0) of bands for all 70 *Vigna* genotypes. Sequential agglomerative hierarical nested (SAHN) clustering was performed on the similarity matrix using Dice coefficient and the unweighted paired group method with arithmetic averages (UPGMA). The correspondence between the RAPD, URP and SSR based similarity coefficient matrices was tested based on correlation analysis and matrix correspondence test (Mantel 1967). The Mantel matrix correspondence test was carried out by using the *MXCOMP* module of *NTSYS Ver 2.1* software (Rohlf 2000). The matrix of similarity coefficient was subjected to 'Eigen' vector analysis to extract the first three most informative principal components analysis (PCA). Utility of a marker for detecting genetic variation was estimated as reported by Powell *et al*.(1996). It includes: *a*) Expected Heterozygosity (Hn) by calculating sum of the squares of allele frequencies, $Hn = 1 - \sum p_i^2$, where pi is the allele frequency for the i^{th} allele (Nei 1973), *b*) Arithmetic mean heterozygosity (H_{av}) was calculated as, $H_{av} = \sum H_n/n$, where n is the number of markers (loci) analyzed, *c*) Effective multiplex ratio ($EMR = n_p\beta$) which is the product of number of polymorphic loci (n_p) in the germplasm analyzed and the fraction of markers that were polymorphic (β), and *d*) Marker index (MI) which is the product of expected heterozygosity (Hav) and effective multiplex ratio were calculated. The resolving

Table 2. Sequences of RAPD, URP and SSR primers used for molecular analysis of 70 genotypes of *Vigna* genus. TNLA - total number of loci amplified, TNB - total number of bands, NPB - number of polymorphic bands, P - percentage of polymorphic band, Rp - resolving power, FER - *ferritin* gene of *Phaseolus vulgaris*; F - forward, R - reverse.

	Primer	Sequence (3'-5')	TNLA	TNB	NPB	P [%]	Rp
RAPD	OPC4	CCGCATCTAC	9	258	258	100	7.47
	OPC11	AAAGCTGCGG	9	240	240	100	6.85
	OPC14	AAGCCTCGTC	9	221	221	100	6.28
	OPC16	CACACTCCAG	8	215	145	67	6.74
	OPE5	TCAGGGAGGT	8	280	280	100	7.99
	OPE11	GGTGACTGTG	5	155	85	55	4.43
	OPF13	GGCTGCAGAA	9	327	257	789	8.94
	OPG13	CTCTCCGCCA	15	216	216	100	6.18
URP	2F	GTGTGCGATCAGTTGCTGGG	6	180	180	100	5.14
	6F	GGCAAGCTGGTGGGAGGTAC	7	215	215	100	6.14
	13R	TACATCGCAAGTGACACACC	8	224	224	100	3.39
	2R	GCCAGCAACTGATCGCACAC	9	177	177	100	5.09
	17R	AATGTGGGCAAGCTGGTGGT	5	244	174	71	5.49
SSR	FER	F-TCGCAAAGTTGCCAGTCAGT R-TAGAAGGAAGGAGGGCCATG	9	211	211	100	6.03

power (Rp) of each primer was calculated as $Rp = \Sigma I_b$, where I_b (band informativeness) takes the value: $1 - [2 \times (0.5 - p)]$, p being the proportion of genotype of different *Vigna* species containing that band (Prevost and Wilkinson 1999).

Results

Level of polymorphism: Among the 70 genotypes of the genus *Vigna* RAPD assay detected 54 % variation amplifying 1912 bands at 72 loci (Table 2). The number of polymorphic bands ranged from 85 to 280 in all the genotypes. The resolving power of RAPD primers ranged from 4.43 to 8.94. Maximum number of loci (15) was amplified with the primer OPG13. Different RAPD markers recorded 55 % to a maximum of 100 % polymorphism in all the genotypes. Five URP markers amplified 1040 bands at 35 loci and detected 72 % variation in all the genotypes (Table 2). The number of polymorphic bands ranged from 177 to 224 with primers URP2R and URP13R, respectively with the best resolving power (6.14 %) of the primer URP6F. Different URP markers recorded 71 - 100 % polymorphism in the *Vigna* genotypes. SSR marker developed from the *ferritin* gene of *P. vulgaris* is a multilocus marker, which amplified nine loci in all the *Vigna* genotypes producing 211 polymorphic bands (Table 2).

Genetic relationships between and within different *Vigna* species: Fourteen DNA markers used for molecular analysis of all the *Vigna* genotypes produced distinct DNA profiles. An example of species-specific fingerprint profile obtained with RAPD marker OPG13 is shown in Fig. 1. This marker characterized all the seven wild and cultivated species of *Vigna* uniquely suggesting an insight for their co-evolution, domestication and inter-specific relationship (Fig. 1). For instance, a specific DNA fragment of 400 bp was found in the genotypes of cultivated species, *Vigna mungo*, *Vigna radiata* and *Vigna unguiculata*. A 700 bp DNA fragment was found in all the genotypes of *Vigna unguiculata*, whereas a unique 800 bp DNA fragment was found in the genotypes belonging to *V. radiata* and *V. mungo* species and their progenitors *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris*, respectively. *V. radiata setulosa* specific band of 3000 bp was also obtained.

Combined data obtained from RAPD, URP and SSR markers on 70 genotypes for the 116 loci were analysed for pairwise genetic similarities based on UPGMA method. Five clusters representing species-specific grouping were obtained (Fig. 2). Cluster I, comprised of all the *Vigna unguiculata* genotypes with 88 % genetic similarity. Cluster II included all the genotypes of *V. mungo* with 90 % genetic similarity and closely related

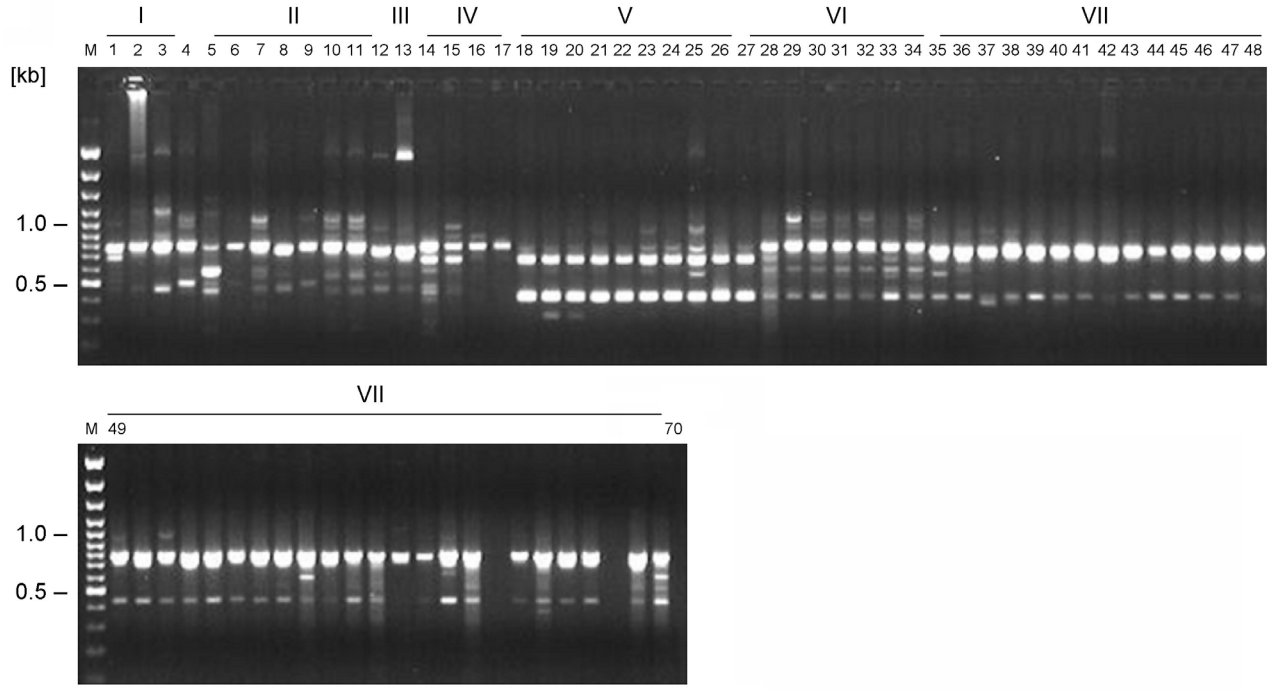

Fig. 1. PCR amplification products obtained with RAPD primer OPG13 from 70 *Vigna* genotypes. Label number represent genotype number (as given in Table 1), where I = *Vigna radiata* var. *sublobata* (genotype 1 - 4), II = *Vigna mungo* var. *silvestris* (genotype 5 - 12), III = *Vigna radiata* var. *setulosa* (genotype 13), IV = *Vigna umbellata* (genotype 14 - 17),V = *Vigna unguiculata* (genotype 18 - 27), VI = *Vigna mungo* (genotype 28 - 34), VII = *Vigna radiata* (genotype 35 - 70) and M = 100 bp DNA marker.

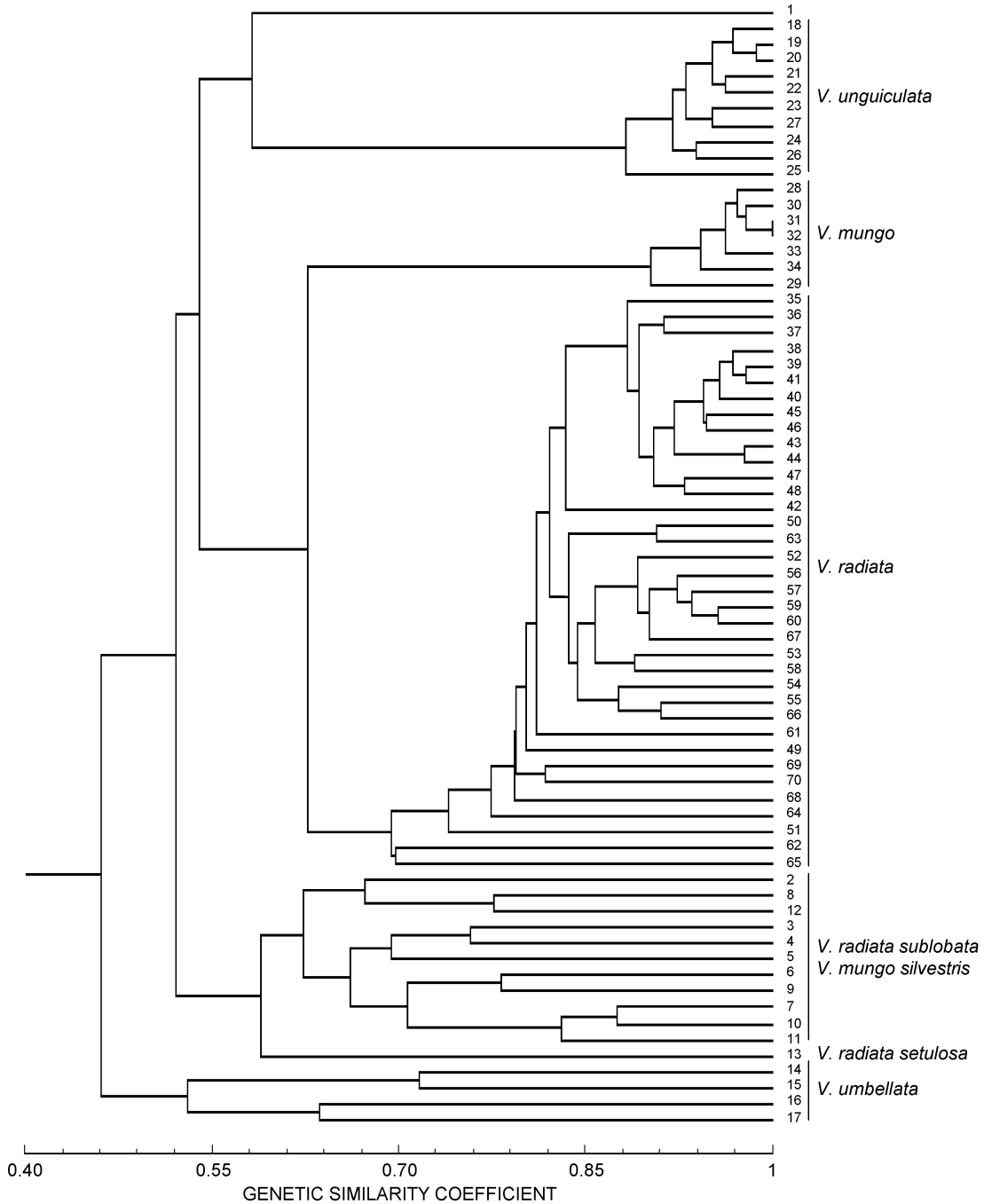

Fig. 2. Dendrogram obtained by analysis of combined data of RAPD, URP and SSR markers from 70 genotypes of *Vigna* species. Analysis was performed with UPGMA. Genotypes numbers are given at the termini of branches. Correlation coefficient r, calculated by Mantel test represents the goodness of fit of the dendrogram.

with *V. radiata* with 69 % genetic similarity. This might be one of the reasons that *V. mungo* is easily hybridized with *V. radiata* and can be used as a donor for MYMV and shattering resistance. A major group (cluster III) of all the genotypes belonging to *V. radiata* was obtained with 70 % genetic similarity. All the genotypes of three wild species, *V. radiata* var. *sublobata*, *V. mungo silvestris* and *V. radiata setulosa* were grouped in Cluster IV with genetic similarity value of 60 %, which also showed 54 % genetic similarity with cultivated *Vigna* species. Of these, *V. radiata* var. *sublobata* (wild) is the progenitor of mungbean and it can provide useful genes for MYMV resistance, preharvest sprouting resistance and tolerance to abiotic stresses (Lawn *et al.* 1987). The genotypes belonging to *V. mungo silvestris*, which is a wild progenitor of *V. mungo*, is a potential source for MYMV resistance. The genotypes of *V. radiata setulosa* (wild) can also be exploited for transferring genes for

profuse podding in cultivated *Vigna* species. All these three species can be crossed with mungbean as the male parent. However, *V. radiata* var. *sublobata* can be utilized both as male and female parents. The cluster V comprises of four genotypes of *Vigna umbellata* exhibiting 53 % genetic similarity (Fig. 2). This species can contribute genes for earliness, early vigour and high biomass. However, successful utilization of genes from this species is yet to be explored for mungbean improvement. Two genotypes PSIC3 and PS2C (numbered as 31 and 32) of *V. mungo* were grouped in cluster II with 100 % genetic similarity. Major clusters of genotypes uniquely differen-tiated wild or cultivated species in the dendrogram obtained from combined marker data of RAPD, URP and SSR markers. Mantel matrix correspondence test revealed high matrix correlation in all the cases with best fit from combined marker data (r = 0.95) followed by URPs (r = 0.94), RAPDs (r = 0.92) and SSR (r = 0.88). The results of principle component analysis (PCA) were comparable to the cluster analysis. The three components explained 63.5, 7.9 and 5.3 % of the total variation and effectively distinguished the seven species within the group. Cultivated *Vigna* genotypes *i.e. V. unguiculata, V. mungo* and *V. radiata* were clustered tightly, while wild genotypes clustered together (Fig. 2.). Genotypes RBL-1 and MNPL3 (numbered as 16 and 17) belonging to *Vigna umbellata* remained to be distinct from other genotypes. This genotype being quite diverse genetically can be used as a parent for intraspecific population improvement. The remaining genotypes showed minimal genetic diversity and all formed one compact cluster.

Marker utility and marker index: Marker utility was assessed from the product of information obtained by measuring different parameters like the average expected heterozygosity, fraction of polymorphic loci and effective multiplex ratio for different marker systems (Table 3).

Table 3. Comparison of different marker systems used in assessing genetic diversity among and between different *Vigna* species. Hav (P) - arithmetic mean heterozygosity, β - fraction of polymorphic loci, EMR - effective multiplex ratio, MI - marker index.

Marker system	Hav (P)	β	EMR	MI
RAPD	0.20	0.88	8.15	1.68
URP	0.25	0.94	6.71	1.60
SSR	0.25	1.00	9.00	2.24

The URPs and SSRs revealed high Hav (0.25) than RAPDs. One single SSR marker revealed high fraction of polymorphic loci (β) and high effective multiplex ratio (9.0) leading to high marker index (MI = 2.24) thus supporting their suitability for the analysis of both interspecific and intraspecific genetic diversity and their use as a potent marker for fine dissection of the intraspecific relationship in *Vigna* species (Wang *et al.* 2004). The URP and RAPD markers yielded comparable results with respect to marker index.

Discussion

While using different marker systems we tested URP markers which were derived from the repeat elements of weedy rice for genetic analysis of different individuals across species (Kang *et al.* 2002). However, these markers have not been used in characterizing *Vigna* species. URP markers are more robust and specific because of the long primer, and high annealing temperatures used for PCR amplification. Our analysis on Mantel matrix correlation also showed that URP markers were better compared to the RAPD and SSR markers derived from beans. These can serve as another marker system in the genetic analysis of *Vigna* spp. where less number of SSR markers is available. Similarly, one SSR marker derived from *ferritin* gene from *P. vulgaris* (Yu *et al.* 2000) was successfully used in molecular analysis of *Vigna* species. It showed that different markers can be used across species while performing genetic analysis at molecular level and also to make across species comparisons.

The critical evaluation of dendrogram revealed minimum diversity in *V. mungo* followed by *V. unguiculata* and *V. radiata* (Fig. 2). The genotypes belonging to cultivated species exhibited higher similarity values as compared to the wild species likes *V. radiata* var. *sublobata*, *V. mungo* var. *silvestris* and *V. radiata* var. *setulosa*. This might be due to the utilization of a few germplasm lines in crop improvement by breeders and the tendency to add desirable and improved characters from a few lines repeatedly, resulting in the narrowing of genetic base of these species. In addition, a large proportion of alleles of higher productivity have been lost in the present population of *Vigna* pulses due to overriding role of natural selection since the domestication of *Vigna* species (Ajibade 2000).

Characterization and assessment of diversity among these *Vigna* species would be of great significance for designing breeding strategies, both for the qualitatative and quantitative improvement. The wild species have great potential for use in mungbean improvement programmes (Srinivas *et al.* 1999). Singh *et al.* (2003) successfully transferred MYMV resistance from *V. umbellata* to *V. radiata* establishing that wild species can be potential sources of desirable traits. The use of these species in hybridization programmes will help in adding more potential variability in cultivated species. In the present study, we were successful to assess the level of inter- and intra-specific diversity and species relationship among different cultivated and wild geno-

types of *Vigna* species. It would therefore, form the basic foundation for further research in crop improvement programmes in pulses. Species-specific markers developed for each species in the present investigation would be of immense significance for any further studies related to the genetic resource characterization and genetic enhancement in this group of nutritive and economically important genus *Vigna.*

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