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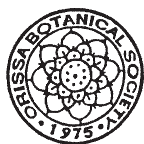

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Analysis of genetic variability and phylogenetic relationships among the species of *Vigna* Savi (Fabaceae) using molecular markers

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ABSTRACT

Genetic diversity and species relationships among 9 species of *Vigna* (*V. aconitifolia, V. adenantha, V. mungo, V. pilosa, V. radiata, V. sublobata, V. trilobata, V. umbellata and V. unguiculata*) with 29 accessions collected from Odisha, Andhra Pradesh and Assam were analysed using 11 RAPD and 16 ISSR markers. A total of 2426 fragments were amplified with both the primer sets, of which 2368 bands were of polymorphic nature. Eighteen unique bands and 58 monomorphic bands were also detected. In the present study, clustering of species in the dendrograms constructed using RAPD, ISSR and combined data was not in complete agreement with the intra-generic classification of Verdcourt (1970) as modified by Marechal *et al.* (1978) but certain species followed the pattern of species placement stated there in. *Vigna sublobata*, considered as a variety of *V. radiata* by many is established here as a distinct species. The phylogenetic tree generated from combined RAPD and ISSR data revealed segregation of taxa into two distinct clusters, one with *Vigna radiata-Vigna mungo* belonging to the sect. Ceratotropis of the subgenus Ceratotropis along with *V. unguiculata* of the sect. Vigna of subgenus Vigna; and the other with members of sect. Leptospron of subgenus Sigmoidotropis and sect. Angulares and sect. Aconitifoliae of the subgenus Ceratotropis. The necessity to reinvestigate the sectional classification of the sub-genus Ceratotropis of genus *Vigna* has been suggested.

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1. Introduction

The legume tribe Phaseoleae (*sensu* Polhill *et al.,* 1981) is the largest Papilionoideae tribe with about 84 genera and 1500 species and economically the most important group containing genera like *Phaseolus, Vigna, Glycine, Cajanus, Clitoria, Macrotyloma, Lablab, Pachyrhizus, Pueraria* and *Psophocarpus*. The genus *Vigna* is comprised of 82 species distributed among 7 subgenera namely, Vigna, Haydonia, Plectotropis, Ceratotropis, Lasiospron, Sigmoidotropis and Macrorhyncha (Verdcourt, 1970; Maréchal *et al*., 1978). The subgenus Ceratotropis consists of 16-17 species including *V. mungo, V. radiata, V. trilobata, V. aconitifolia, V. umbellata*, which are distributed across Asia and constitute an economically important group of cultivated and wild species with rich diversity in India (Arora, 1985; Babu *et al*., 1985). Based on cross compatibility studies and seedling characteristics, Tateishi (1996) recognised three isolated genepools in Asian *Vigna i. e. angularis-umbellata* (azuki bean group), *radiata-mungo* (mungbean group) and *aconitifoila-trilobata* (mothbean group), which was supported by isozyme and molecular studies (Jaaska and Jaaska, 1990; Kaga *et al*., 1996. Tomooka *et al*. (2000) proposed a revised list of taxa in the subgenus Ceratotropis and suggested three groups, giving them taxonomic rank as section *Angulares* (azuki bean group), *Radiateae* (mungbean group) and *Aconitifoliae* (mothbean group) and an undetermined section. Subgenus *Vigna* is by far the most species-rich subgenus, most of which are endemic to Africa including *V. unguiculata* and *V. subterranea*. *Vigna. adenantha* comes under the subgenus Sigmoidotropis, which is of Neotropical origin and is considered primitive.

The genetic diversity and molecular phylogeny of different species of *Vigna* has been studied by a number of workers in many parts of the world but with more emphasis on

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cultivated species like mung bean, azuki bean, cowpea and moth bean (Kaga *et al*., 1996; Tomooka *et al*., 2002; Goel *et al*., 2002; Souframanien and Gopalakrishna, 2004; Bisht *et al*., 2005; Seehalak *et al*., 2006; Simon *et al*., 2007; Vir *et al*., 2010; Tantasawat *et al*., 2010; Javadi *et al*., 2011). Only a few of them have analysed the genetic diversity and phylogeny of wild *Vigna* species along with accessions of cultivated crops. In the present study the genetic diversity and phylogenetic relationship among 9 species and 29 accessions of *Vigna* have been studied using 11 RAPD and 16 ISSR primers.

2. Materials and methods

2.1 Plant materials

The viable seed samples of 29 accessions belonging to 9 species of *Vigna* (*V. aconitifolia, V. adenantha, V. mungo, V. pilosa, V. radiata, V. sublobata, V. trilobata, V. umbellata and V. unguiculata*) were collected from forests and institutions of Odisha, Andhra Pradesh and Assam. The accession number, locality of collection and abbreviation used for each of the taxon is shown in Table 1. The seed materials were geminated in pro-trays under greenhouse conditions at Regional Plant Resource Centre, Bhubaneswar and the tender leaves were used for DNA extraction for molecular analyses. The herbarium specimens have been deposited in the herbarium of RPRC, Bhubaneswar Odisha (India).

2.2 Isolation, purification and quantification of genomic DNA

Genomic DNA was extracted from the leaf tissues using the modified CTAB (cetyl-trimethyl-ammonium-bromide) protocol (Doyle and Doyle, 1990) with little modification. Two grams of leaf tissues from young seedlings were ground with extraction buffer composed of 100 mM sodium acetate (pH 4.8), 500 mM NaCl, 20 mM EDTA (pH 8.0); 100 mM Tris (pH 8.0); 2% Polyvinyl pyrollidone (PVP) and 2% CTAB. The precipitated DNA was washed twice with 70% ethanol, stored in micro centrifuge tube and dried in DNA-mini vacuum dryer (DNA Mini, Germany). The dried DNA was dissolved in excess amount of $T_{10}E_1$ buffer (Tris-Cl 10mM, EDTA 1mM pH 8).

The RNA was removed by the standard technique of RNase-A treatment, incubation, washing with chloroform: isoamyl alcohol (24:1) and centrifugation. The dried DNA

Table 1
Details of the plant samples used for the study

Sl. No.	Species	No of accessions studied	Place(s) of collection	Code used
1	*Vigna trilobata*	4	Bhubaneswar	Vt BBS1 &2
			Khurda	Vt KUR
			Kandhamal	Vt KML
2	*Vigna sublobata*	5	Khurda	Vs KUR
			Nayagarh	Vs NGD
			Kalahandi	Vs KND
			Kandhamal	Vs KML1&2
3	*Vigna umbellata*	2	Kandhamal	Vu KML
			Nayagarh	Vu NGD
4	*Vigna radiata*	4	Ganjam	Vrad GN
			Bhubaneswar	Vrad BBS
			Nayagarh	Vrad NGD 1&2
5	*Vigna mungo*	7	Ganjam	Vmun GN1&2
			Silchar, Assam	Vmun ASM
			Bhubaneswar	Vmun BBS
			Nayagarh	Vmun NGD1,2&3
6	*Vigna pilosa*	1	Khurda	Vpil
7	*Vigna unguiculata*	2	Nayagarh	Vung LONG
				Vung SHORT
8	*Vigna aconitifolia*	2	Anantapur, Andhra Pradesh	Va1, Va2
9	*Vigna adenantha*	2	Bhubaneswar	Vad1, Vad2

was dissolved in minimum amount of $T_{10}E_1$ buffer (pH-8.0). The quality and quantity of DNA was measured by UV-VIS Spectrophotometer (Bio Photometer, Eppendorf, Germany). For final checking of the quality as well as quantity of DNA, the DNA was loaded in 0.8% agarose gel stained with Ethidium Bromide (0.5µg/ml) and one side diluted uncut ë DNA as standard and electrophoresed. After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 100 ng/ml. Again the DNA was loaded in 0.8% agarose gel stained with Ethidium Bromide (0.5µg/ml) and electrophoresed to see the uniformity of concentration in all the samples. Finally, it was diluted to 25ng/µl with $T_{10}E_1$ buffer and the DNA samples were stored at -20ºC in a deep freezer for PCR analysis.

2.3 *Random amplified polymorphic DNA (RAPD) analysis*

Prior to polymerase chain reaction (PCR), random decamer primers (Operon Technologies, Alameda, USA) were dissolved in double sterilized $T_{10}E_1$ buffer (pH 8.0) to the working concentration of 15 ng/ml. Twenty best selected primers as per the reproducibility and amplification pattern from A, D, N, P, S, T and AF series (Operon Technologies, Alameda, CA.) were selected for RAPD analysis (Table 2). The RAPD analysis was performed as per the methodology described by Williams *et al.* (1990). Each amplification reaction mixture of 25 ml volume contained 2.5 ml of 10X assay buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 200 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Fermantas, Lithuania) 15 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25 ng of template DNA. The amplification reaction was carried out in GeneAmp PCR System 9700 (Applied Biosystems, Germany). The amplification was performed in three steps PCR. Initial denaturation of the template DNA was carried out at 94 ^{0}C for 5 min for one cycle. The second step was carried out for 42 cycles and each cycle consisting of three temperature steps i.e. one min at 92 ^{0}C for denaturation of template, one min at 37^{0}C for primer annealing followed by two min at 72^{0}C for primer extension. The final step consisted of only one cycle i.e. 7 min at 72 ^{0}C for complete polymerization. The soak temperature was 4^{0}C. After the completion of the PCR 2.5 ml of 6X loading dye (MBI Fermentas, Lithuania) was added to the amplified products and were stored at –20^{0}C till further use.

2.4 *Inter simple sequence repeat (ISSR) analysis*

For the present investigation twenty (20) anchored and non-anchored microsatellites were used as primers. These simple sequence repeats were synthesized and procured from Genei (Bangalore Genei Pvt. Ltd, Bangalore, India). The details of their sequence, base pairs and codes used are given in the Table 3. The ISSR analysis was performed as per the methodology given by Zietkiewicz *et al.* (1994). Each amplification reaction mixture of 25 ml contained 20ng of template DNA, 2.5ml of 10X assay buffer (100mM Tris-HCl pH 8.3, 0.5M KCl and 0.01% gelatin), 1.5mM $MgCl_2$, 200mm each of dNTPs, 44ng of primer and

Table 2
Details of RAPD primers used and bands amplified

Primer Name	Sequence	Total no. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% of Polymorphic Bands (PPB)	Resolving Power	Primer Index
OPA 02	5’TGCCGAGCTG3’	48	48	0	2	100	3.3	2.00
OPD 20	5’ACCCGGTCAC3’	86	86	0	0	100	5.93	2.95
OPN 02	5’ACCAGGGGCA3’	66	66	0	0	100	4.55	2.32
OPA 03	5’AGTCAGCCAC3’	82	82	0	3	100	5.65	2.93
OPN 06	5’GAGACGCACA3’	80	80	0	1	100	5.51	3.29
OPN 11	5’TCGCCGCAAA3’	66	66	0	2	100	4.55	3.02
OPN 12	5’CACAGACACC3’	119	119	0	0	100	8.20	4.38
OPN 16	5’AAGCGACCTG3’	63	63	0	3	100	4.34	2.58
OPA 10	5’GTGATCGCAG3’	59	59	0	1	100	4.06	1.14
OPA 18	5’AGGTGACCGT3’	91	91	0	1	100	6.27	3.82
OPN 04	5’GACCGACCCA3’	34	5	29	0	14.70588	2.34	0.28
TOTAL		794	765	29	13	96.34761	54.75	28.74

Table 3
Details of ISSR primers used and bands amplified

Primer Name	Sequence	Total no. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% of Polymorphic Bands (PPB)	Resolving Power	Primer Index
AGG6	5'AGGAGGAGGAGGAGGAGG3'	119	119	0	1	100	8.20	2.60
AK 10	5'CTCTCTCTCTCTCTCTA3'	80	80	0	0	100	5.51	3.42
AK 7	5'GTGGTGGTGGTGGTG3'	68	68	0	0	100	4.68	1.47
CCA 5	5'CCACCACCACCACCA3'	89	89	0	0	100	6.13	1.82
GA 10	5'GAGAGAGAGAGAGAGAGAGA3'	121	121	0	0	100	8.34	3.42
GA9T	5'GAGAGAGAGAGAGAGAGAT3'	64	64	0	1	100	4.41	2.26
GA9TA	5'GAGAGAGAGAGAGAGAGATA3'	165	165	0	0	100	11.37	5.51
Oligo 1 a	5'AGAGAGAGAGAGAGAGG3'	87	87	0	1	100	6.00	2.56
Oligo 1 b	5'AGAGAGAGAGAGAGAGC3'	118	118	0	2	100	8.13	2.37
Oligo 2 a	5'AGAGAGAGAGAGAGAG3'	74	74	0	0	100	5.10	2.00
Oligo 2 b	5'GAGAGAGAGAGAGAGAG3'	59	59	0	0	100	4.06	1.92
Oligo 3 b	5'GACAGACAGACAGACA3'	65	65	0	0	100	4.48	2.70
Oligo 5b	5'GACAGACAGACAGACAG3'	184	184	0	0	100	12.68	3.58
Oligo 8A	5'CTCTCTCTCTCTCTCTCTG3'	64	64	0	0	100	4.41	2.80
Oligo 9A	5'GCTCTCTCTCTCTCTCT3'	135	135	0	0	100	9.31	4.49
TGA 9	5'TGAGAGAGAGAGAGAGAGA3'	140	111	29	0	79.28571	9.65	3.53
TOTAL		1632	1603	29	5	98.22304	112.55	46.52

0.5 U Taq DNA polymerase. The amplification was carried out in a PCR system. (GeneAmp PCR System 9700, Applied Biosystems). The first cycle consisted of denaturation of template DNA at 94^{0}C for 5 min, primer annealing at specific temperature for particular primer (as indicated in the Table 2) for 1 min and primer extension at 72 ^{0}C for 2 min. In the subsequent 42 cycles the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was maintained same as in the first cycle. The last cycle consisted of only primer extension at 72^{0}C for 7 min. the amplified products were resolved in 2% agarose gel stained with ethidium bromide.

The PCR products for RAPD were separated in 1.4 % agarose gel while those of the ISSR products were resolved in 2 % agarose gel. After electrophoresis, the gel was visualized under the UV-transilluminator (BioRad, USA) and photographed. The gel was also documented in Gel Doc 2000 (BioRad, USA) for scoring the bands.

2.5 *Scoring and analysis of data*

The data was scored as '1' for the presence and '0' for the absence of the band for each primer genotype combination for RAPD and ISSR (Gherardi *et al.*, 1998). Resolving power of the RAPD was calculated as per Prevost and Wilkinson (1999). Resolving power is: $Rp=\Sigma IB$ (IB=Band informativeness= $1-[2x(0.5-P)]$, P is the proportion of the species containing the band. Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a phylogram based on similarity coefficients generated by Unweighted Pair Group Method using Arithmetic averages (UPGMA) (Sneath and Sokal, 1973), and the SAHN (Sequential Agglomerative Hierarchial and Nested) clustering was obtained. The entire analysis was performed using the statistical package NTSYS-pc 2.02e (Rohlf, 2000).

3. Results

3.1 *Assessment of genomic relationship by RAPD analysis*

Eleven RAPD primers used in the experiment led to amplification of 794 bands. Of these 765 fragments were polymorphic in nature. The pattern of banding is shown in Fig. 1. The details of primers used and bands amplified are given in Table 2. The highest numbers of fragments (119) were amplified by the primer OPN 12 and lowest (34) by the

primers OPN 4. Maximum total number of 42 bands were amplified in *Vigna adnantha* (Vad1) and minimum (17) in *Vigna aconitifolia* (Va1).

The tree generated based on RAPD data placed *Vigna pilosa* in a separate position with only 19% similarity with the rest of the taxa (Fig. 2). The other 28 species and accessions were divided into 2 distinct clusters, the smaller being the two accessions of *Vigna aconitifolia* (Vac1 & Vac2). The larger cluster was bifurcated into two groups of 14 and 12 accessions with a similarity coefficient of 0.24. The clade with 12 taxa contained accessions of *Vigna sublobata, Vigna umbellata, Vigna trilobata* and *Vigna adenantha* and the other had species like *Vigna radiata, Vigna mungo* and *Vigna unguiculata* and other accessions. With the former group, further division to *Vigna sublobata-Vigna umbellata* group and *Vigna trilobata-Vigna adenantha* group, could be noticed and both these clusters shared about 29% genetic similarity. Similarly, in the late cluster of 14 taxa, 2 accessions of *Vigna unguiculata* were the first to come out of the clade and had a similarity of about 28%. Besides, the rest of the accessions segregated to *Vigna radiata* and *Vigna mungo* accessions and both the species had 43% common genomic characters. However, a single accession of *Vigna trilobata* got mingled up with the accessions of *Vigna radiata*.

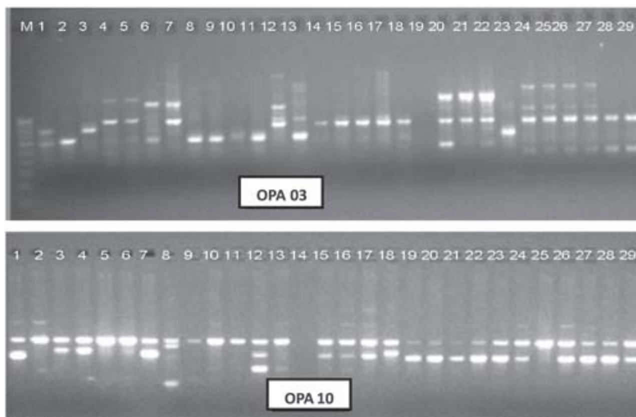

Fig. 1. RAPD banding pattern in different species of *Vigna* with use of the primers OPA03 (LKA 2) and OPA10 (LKA 5). M-marker.

Similarity coefficient calculated on the basis of RAPD data indicated that the accessions of *Vigna radiata* GN and BBS were closely related with a similarity coefficient of 0.933. *Vigna pilosa* and *Vigna sublobata*-KUR showed only 8% similarity and thus distantly located in the dendrogram. All species of the genus *Vigna* accessions within them had an average similarity of 0.434.

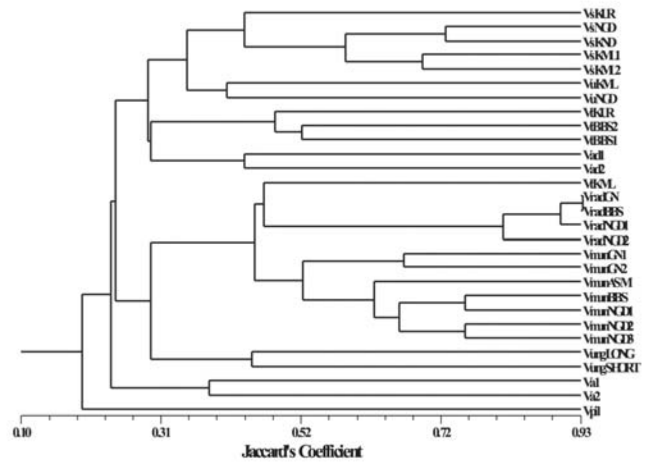

Fig. 2. Dendrogram showing genetic relationship among different species of *Vigna* as revealed from RAPD analysis.

3.2 Genome analysis using ISSR markers

The pattern of banding with the use of 16 selected ISSR primers is presented in Fig. 3. In this case, out of a total of 1632 bands amplified, 1598 bands were polymorphic and 29 monomorhic in nature; only 5 were private ones (Table 3). A maximum of 184 bands were amplified with the primer (GACA)4G and the lowest 59, in case of primer G(AG)8. The highest resolving power (12.689) and Primer Index (5.517) were recorded for the primer (GACA)4G and (GA)9 TA respectively. In *Vigna radiata*-BBS a total of 70 bands were amplified, which was higher among all the species and accessions.

It was noticed that the two subspecies of *Vigna unguiculata* got separated in the cladogram (Fig. 4) in the first place from all other species with a similarity of 32%. The rest 8 species formed two major groups, one with *Vigna radiate, Vigna mungo* and their genotypes and other six species in the second cluster. The smaller cluster had 4 accession of *Vigna radiata* and 7 accessions of *Vigna mungo*, which were closely located justifying their entity as biological species. Most of the accessions of *Vigna radiata* and *V. mungo* from a particular geographical area tended to come together. Within the large group containing 16 accessions of 6 species of *Vigna*, two genotypes of *Vigna aconitifolia* were out-grouped in the dendrogram at a similarity of 37%. After exclusion of *Vigna aconitifolia*, the rest of the taxa were separated into 2 clusters of 7 accessions each; one containing *Vigna sublobata, Vigna umbellata* and *Vigna pilosa* and the other with *Vigna adenantha, Vigna trilobata* and lone genotype of *Vigna umbellata*. *Vigna adenantha* got separated from *Vigna trilobata* group at a similarity level of about 42%. All accessions of *Vigna sublobata* and *Vigna trilobata* formed tight clusters within their respective sub-clusters in the dendrogram.

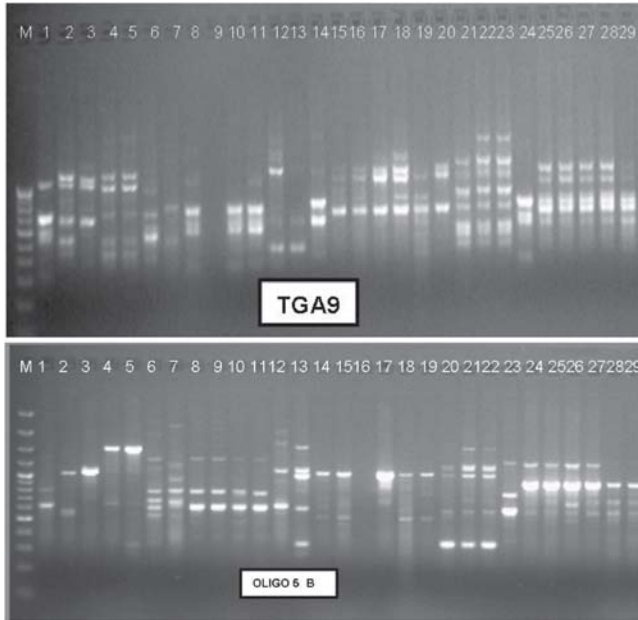

Fig. 3. ISSR banding pattern in species of *Vigna* with the primers TGA9 and Oligo 5 B. M- marker.

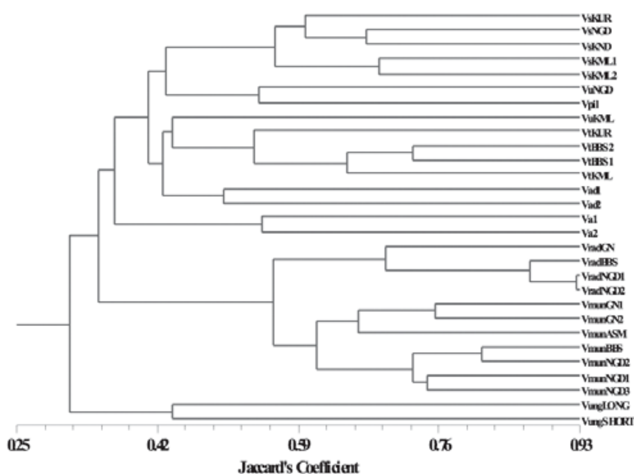

Fig.4. Phylogenetic tree showing genetic relationship among different species of *Vigna* based on ISSR data.

3.3 Analysis of phylogeny using RAPD and ISSR combined markers

A total of 2426 bands were produced with the use of 27 ISSR and RAPD primers, of which 2368 bands were of polymorphic nature. Eighteen unique and 58 monomorphic bands were also detected. *Vigna adenantha* (Vad1) resolved highest number of fragments (109), while *Vigna aconitifolia* (Vac1) produced the least number (63) of bands.

The tree generated from combined RAPD and ISSR markers data (Fig.5) revealed similar type of relationship among the species and accessions as found with ISSR analysis with little deviation. Here, two distinct clusters were observed, one with 16 accessions of *Vigna sublobata, Vigna umbellata, Vigna trilobata, Vigna adenantha, Vigna pilosa* and *Vigna aconitifolia* and the other with 13 accessions of *Vigna radiata, Vigna mungo* and *Vigna unguiculata*. Both the two major clusters had a similarity value of 0.31. Within *Vigna radiata-Vigna mungo-Vigna unguiculata* group, the two subspecies of *Vigna unguiculata* got isolated. Further, 7 genotypes of *Vigna mungo* and 4 genotypes of *Vigna radiata* formed 2 distinct groups sharing a similarity of about 53%.

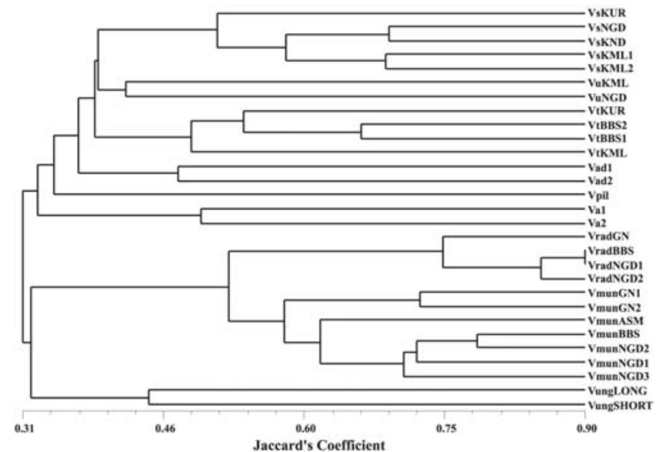

Fig 5. Dendrogram tree showing genetic relationship among *Vigna* species based on combination of RAPD and ISSR analysis.

Among the other group containing 16 accessions of 6 *Vigna* species, *V. aconitifolia* was the first to go as an out-group followed by *Vigna pilosa*. While *Vigna aconitifolia* had a genetic similarity of 33% with other members in the cluster, *Vigna pilosa* was similar by 35%. The clade was further sub-divided and 2 accessions of *Vigna adenantha* occupied a distinct place. Subsequently, accessions of *Vigna trilobata* and *Vigna umbellata* came out of the cluster leaving the 5 accessions of *Vigna sublobata* as a sub-group. *Vigna trilobata* accessions shared a similarity of 39% with *Vigna sublobata-Vigna umbellata* complex. Within each cluster accessions of a particular collection locality had closer genetic affinity among them.

4. Discussion

A total of 2426 fragments were amplified with both the primer sets, of which 2368 bands were of polymorphic nature. Eighteen unique bands and 58 monomorphic bands were also detected. One accession of *Vigna adenantha* was found to be highly polymorphic (109) and least polymorphism (63) was noted in case of a *Vigna aconitifolia*.

The phylogram generated using RAPD data clearly segregated *Vigna pilosa* from all other species and shared a similarity of mere 19%. The species *V. pilosa* is morphologically dissimilar from other species of the genus in its climbing habit and velvety nature of pods. Pandiyan *et al*. (2012) undertook diversity analysis of *Vigna* species through morphological markers and established *V. pilosa* as a divergent species. The above species was treated under a separate subgenus *Dolichovigna* and found it to be taxonomically quite distinct from other taxa (Bisht *et al.,* 2005) and later *Dolichovigna* was removed from *Vigna* and placed as a subgenus in *Dysolobium*. Marechal *et al*. (1978) transferred *V. pilosa* to the genus *Dysolobium* (Benth.) Prain and the species is better known today under the name *Dysolobium pilosum* (Willd.) Marechal (cf. Sanjappa, 1992). The result of RAPD analysis of the present work supported this view. However, the data obtained from ISSR and combined RAPD and ISSR markers could not be corroborated with the findings based on morphological characteristics (Marechal *et al*., 1978; Sanjappa, 1992; Bisht *et al*., 2005).

The intra-generic classification of Verdcourt (1970) as modified by Maréchal *et al.* (1978) recognised 7 subgenera namely, Vigna, Haydonia, Plectotropis, Ceratotropis, Lasiospron, Sigmoidotropis and Macrorhyncha. Of the *Vigna* species selected for the present work, the subgenus *Vigna* was represented by *Vigna unguiculata* and subgenus Ceratotropis by six species namely, *V. mungo, V. radiata, V. sublobata, V. aconitifolia, V. trilobata* and *V. umbellata*. *Vigna adenatha* was the only species under the subgenus Sigmoidotropis. During the present study, clustering of species in the dendrograms constructed using RAPD, ISSR and combined data was not in complete agreement with the intra-generic classification stated above but certain species followed the pattern of species placement stated above. On analysis of ISSR marker data, two subspecies of *Vigna unguiculata* (*V. unguiculata* ssp. *unguiculata* and *V. unguiculata* ssp. *cylindrica*) belonging to the subgenus Vigna got separated from members of two other subgenera viz. Ceratotropis and Sigmoidotropis in the phylogenetic tree.

The tree generated from combined RAPD and ISSR markers data revealed grouping of taxa into two distinct clusters, one with *Vigna radiata-Vigna mungo* belonging to the sect. Ceratotropis of the subgenus Ceratotropis along with *V. unguiculata* of the sect. Vigna of subgenus Vigna; and the other with members of sect. Leptospron of subgenus Sigmoidotropis and sect. Angulares and sect. Aconitifoliae of the subgenus Ceratotropis. Though both *V. aconitifolia* and *V. trilobata* were members of the sect. Aconitifoliae of the subgenus Ceratotropis, they were placed remotely. Using ISSR polymorphism, Ajibade *et al.* (2000) have also observed clear separation of *C. aconitifolia* from other species of *Vigna.*

Vigna sublobata, which is considered as a variety of *V. radiata* (Verdcourt, 1970; Saini and Jawali, 2009; Bisht *et al*., 2005; Dikshit *et al*., 2005), was found to be quite distinct from *V. radiata* and did not come even in the same clade. This authenticates the view of Bairiganjan *et al*. (1985) in treating this widely accepted variety as a true species. Morphological distinction, analysis of seed protein content, amino acid composition and crossability experiments by Babu *et al*. (1985) established *V. sublobata* as a putative progenitor of *V. mungo* and as remotely related to *V. radiata*. The F1 hybrid between *V. mungo* and *V. sublobata* and *V. radiata* and *V. sublobata* were observed to be sterile pointing at sterility barriers among these three species (Babu *et al*., 1985). UPGMA tree based on RAPD profiles placed *V. mungo*, *V. radiata* and *V. radiata* var. *sublobata* separately and were considered distinct taxonomic entities. Undal *et al*. (2011) found *V. radiata* and *V. radiata* var. *sublobata* (=*V. sublobata*) in two separate clusters in the dendrogram constructed on the basis of RAPD analysis further strengthened the above view.

Recently, the subgenus Ceratotropis has been divided into three sections Aconitifoliae, Angulares and Ceratotropis (Tomooka *et al*., 2002). However, in the present study using RAPD and ISSR in combination, *V. radiata* and *V. mungo* belonging to the section Ceratotropis came in one cluster but *V. sublobata* (*V. radiata* var. *sublobata*) of the same section formed a different clade with members of other sections in the phylogram. *V. aconitifolia* and *V. trlobata* of the section Aconitifoliae were grouped in a larger cluster but away from each other and intermingled with other taxa of the section Ceratotropis (*V. sublobata*) and Angulares (*V. umbellata*) and even unrelated species of subgenus Sigmoidotropis (*V. adenantha*). Therefore, the sectional classification of the subgenus Ceratotropis could not be resolved using either RAPD or ISSR markers. As remarked by Vir *et al.* (2010), the inability of ISSR markers to reveal sectional relationships could either be due to their inability to reflect the divergence within taxa owing to different evolutionary rates or alternatively, the earlier classification proposed (Verdcourt, 1970; Marechal *et al*., 1978; Tateishi, 1996) needs to be reinvestigated. Saini and Jawali (2009) studied the molecular evolution of 5S rDNA gene unit among 10 *Vigna* species belonging to the subgenus Ceratotropis and observed that the species relationship are not in agreement with sectional classification (Tomooka *et al*., 2002). In the present study, similar observations have been made and this necessitates reinvestigation of the sectional classification of the sub-genus Ceratotropis of genus *Vigna*.

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