Genet Resour Crop Evol (2009) 56:783–795
DOI 10.1007/s10722-008-9401-3

RESEARCH ARTICLE

Analysis of population substructure, genetic differentiation and phylogenetic relationships among selected Asiatic *Vigna* Savi species

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Received: 24 June 2008 / Accepted: 10 December 2008 / Published online: 28 January 2009

Abstract Random amplified polymorphic DNA markers were used to study sub-structure and genetic differentiation amongst 31 populations (seven cultivated and 24 wild populations) belonging to 14 Asiatic *Vigna* species. Ten pre-selected RAPD primers generated 152 polymorphic amplification products. Estimates of polymorphism indices were higher for the wild taxa in comparison to the cultivated forms. F_{ST} values between populations ranged from 0.111 to 0.801 and Nei's genetic diversity values between and within species varied from 0.26 to 0.70 and 0.04 to 0.56 respectively. The high F_{ST} and F_{CT} values indicated strong subdivision of populations and high differentiation among species. Analysis of molecular variance was performed by grouping the populations conforming to specific species. AMOVA was also performed separately to better resolve the differentiation of species within mungo–radiata complex. Molecular phylogenetic relationships amongst the species of *radiata–mungo* complex; namely, black gram (*V. mungo* (L.) Hepper), green gram (*V. radiata* (L.) Wilczek), *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*, *V. mungo* var. *silvestris* and *V. hainiana*, were studied through cluster analyses. Two distinct groups were recognized within the complex, with population samples of *V. hainiana* forming one cluster. Further, *V. hainiana* appeared to be equidistant to both *V. radiata* and *V. mungo*.

Keywords Asiatic *Vigna* · Population substructure · RAPD · Species differentiation

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Introduction

The genus *Vigna* Savi has approximately 150 species that are grouped into seven subgenera. Asiatic grams are assigned to subgenus *Ceratotropis* (Piper.) Verdc. with 16–17 recognized species (Verdcourt 1970; Maréchal et al. 1978; Tateishi 1996). Of these eight species, namely, *V. radiata*, *V. mungo*, *V. angularis*, *V. aconitifolia*, *V. umbellata*, *V. trilobata*, *V. trinervia* and *V. reflexo-pilosa* var. *glabra* are used as human and animal food. Thus, Asiatic *Vigna* constitutes an economically important group of cultivated and wild species, of which rich diversity occurs in India (Arora 1985; Babu et al. 1985).

The taxonomy of *Vigna* species is complex and needs detailed analysis, as the estimation of genetic diversity and delineation of several important taxa

remain uncertain till date. Few studies have addressed genetic variation in most important Asiatic pulse yielding *Vigna* species, namely *V. radiata* and *V. mungo*, in relation to the wild forms which have been designated as distinct species or alternatively as botanical varieties of the cultivated species by different workers. For a long period, *V. radiata* var. *sublobata* had been considered the common ancestor of green gram and black gram. However, Lukoki et al. (1980) and Chandel et al. (1984) recognized divergence of both species before their domestication, and reported that *V. radiata* and *V. mungo* were domesticated from two very different taxa, namely, *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris*. Babu et al. (1985) did not recognize such varietal status for wild forms; instead they described all these wild forms as one species, namely, *V. sublobata* on the basis of morphological similarities observed in the natural populations occurring in Indian subcontinent and also, described a new species, *V. hainiana*, to delineate more primitive and wild forms with greater diversity for morphological traits in comparison to the members of the *V. sublobata* species.

Presence of gaps in the available information has necessitated the use of molecular markers for the analysis of the population substructure, genetic diversity and phylogenetic relationships amongst selected species of the genus *Vigna*. The present study was formulated with the following objectives: (a) to study population substructure and genetic diversity in selected Asiatic *Vigna* species (b) to investigate status and distinctiveness of *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa*, *V. mungo* var. *silvestris* and *V. hainiana* (c) to investigate relationships of the wild forms with *V. radiata* and *V. mungo*. These objectives are addressed in the selected *Vigna* species through RAPD analysis.

Materials and methods

Plant material and DNA extraction

A total of 31 selected populations of 14 *Vigna* species were analyzed in the study (Table 1). Total genomic DNA was extracted from 15-day-old individual etiolated seedlings using CTAB extraction procedure (Saghai-Maroof et al. 1984). DNA was treated with Bovine Pancreatic RNase A and extracted once with phenol:chloroform:isoamylalcohol (25:24:1 v/v) and twice with chloroform:isoamylalcohol (24:1 v/v). After precipitation with chilled absolute ethanol, two washes of 70% ethanol were given. The concentration of DNA samples was estimated using DNA flourometer (Hoefer scientific, San Francisco, USA) employing Hoechst 33258 as DNA intercalating dye (Brunk et al. 1979). A diluted stock of 20 ng/μl DNA was used for setting up PCR amplification reactions.

Primer selection

Forty-two deca-nucleotide RAPD primers from kits OPA, OPB, OPC, OPD and OPH of Operon Technologies (USA) were screened for repeatability and scorability using two samples each of *V. radiata* and *V. mungo*. Out of these 42 primers, 10 primers yielding repeatable, good and polymorphic amplification products were selected. The nucleotide sequences of these primers and characteristics of amplification products recorded are listed in Table 2. The PCR amplification reaction mixture contained 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM tris HCl (pH 9), 0.1% Triton X-100, 0.2 mM dNTPs, 1 unit Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.4 μM primer and 20 ng genomic DNA in a reaction volume of 25 μl. Amplification was performed using GeneAmp 9600 Thermal Cycler (Perkin Elmer Cetus, Norwak, CT, USA) with the following temperature profile: a pre-denaturation step of 3 min at 94°C, amplification reactions were cycled 40 times at 94°C for 1 min, 32°C for 1 min and 72°C for 1 min, a final extension step was allowed for 10 min at 72°C. The amplification products were separated in 1.8% agarose gel by electrophoresis in 1× TAE buffer, pH 8.0. The 1 kb DNA marker ladder (MBI Fermentas, Germany) was used as molecular weight standard. After electrophoresis, the gels were stained with ethidium bromide, viewed under UV light and photographed with Polaroid 667 film.

Data scoring and analysis

All gel patterns were scored for presence and absence of RAPD fragments, and data was entered into a binary data matrix as discrete variables (1 for

Table 1 List of 31 *Vigna* accessions analyzed along with the place of collection and status of the material

S. No	Botanical name	Accession no.	Code	Wild/ cultivated	Source of the material
1.	*V. angularis* (Willd.) Ohwi et Ohashi	Vangul-1	Vangul	Cultivated	Meghalaya
2.	*V. dalzelliana* (O. Kuntze) Verdc.	BBL69-2K	BBL69_2Kdal	Wild	Ambaghat, Maharashtra
3.	*V. hainiana* Babu, Gopinathan et Sharma	TCR24	TCR24hai	Wild	Kalwara, Madhya Pradesh
4.	*V. hainiana* Babu, Gopinathan et Sharma	TCR26	TCR26hai	Wild	Sheshasahi Hills, Madhya Pradesh
5.	*V. hainiana* Babu, Gopinathan et Sharma	TCR27	TCR27hai	Wild	Mandu, Madhya Pradesh
6.	*V. hainiana* Babu, Gopinathan et Sharma	TCR29	TCR29hai	Wild	Kachari, Madhya Pradesh
7.	*V. hainiana* Babu, Gopinathan et Sharma	BB6-2K	BB6_2Khai	Wild	Bherughat, Madhya Pradesh
8.	*V. hainiana* Babu, Gopinathan et Sharma	BBL18-2K	BB18_2Khai	Wild	Amaravati, Maharashtra
9.	*V. hainiana* Babu, Gopinathan et Sharma	BBD7-01B	BBD7_01Bhai	Wild	Gajapati, Orissa
10.	*V. hainiana* Babu, Gopinathan et Sharma	BB25-01B	BB25_01Bhai	Wild	Bilaspur, Chhattisgarh
11.	*V. minima* (Roxb.) Ohwi et Ohashi	BBL79-2K	BBL79_2Kmin	Wild	Asanore, Goa
12.	*V. mungo* (L.) Hepper var. *silvestris* Lukoki, Marechal et Otoul	BBL40-2K	BBL40_2Kmsil	Wild	Khapoli, Maharashtra
13.	*V. mungo* (L.) Hepper var. *silvestris* Lukoki, Marechal et Otoul	BBL50-2K	BBL50_2Kmsil	Wild	Murud, Maharashtra
14.	*V. mungo* (L.) Hepper var. *silvestris* Lukoki, Marechal et Otoul	BBL55-2K	BBL55_2Kmsil	Wild	Khedaghat, Maharashtra
15.	*V. mungo* (L.) Hepper var. *silvestris* Lukoki, Marechal et Otoul	BBL60-2K	BBL60_2Kmsil	Wild	Dervan, Maharashtra
16.	*V. mungo* (L.) Hepper var. *silvestris* Lukoki, Marechal et Otoul	BBL83-2K	BBL83_2Kmsil	Wild	Rajapur, Maharashtra
17.	*V. mungo* (L.) Hepper	PPU14	Mungo	Cultivated	Kanpur, Uttar Pradesh
18.	*V. mungo* (L.) Hepper	NG2119	NG2119mun	Cultivated	Uttar Pradesh
19.	*V. radiata* (L.) Wilczek var. *setulosa* (Dalzell) Ohwi et Ohashi	BBL69	BBL69s	Wild	Ambaghat, Maharashtra
20.	*V. radiata* (L.) Wilczek var. *setulosa* (Dalzell) Ohwi et Ohashi	BBL72-2K	BBL72_2Ks	Wild	Radhanagari, Maharashtra
21.	*V. radiata* (L.) Wilczek var. *sublobata* (Roxb.) Verdcourt	TCR11	TCR11sub	Wild	Payyalore, Maharashtra
22.	*V. radiata* (L.) Wilczek var. *sublobata* (Roxb.) Verdcourt	TCR62	TCR62sub	Wild	Ratnagiri, Maharashtra
23.	*V. radiata* (L.) Wilczek var. *sublobata* (Roxb.) Verdcourt	BBL64-2K	BBL64_2Ksub	Wild	Ratnagiri, Maharashtra
24.	*V. radiata* (L.) Wilczek	Pusa105	Pusa105rad	Cultivated	IARI, New Delhi
25.	*V. radiata* (L.) Wilczek	UPM92	UPM92rad	Cultivated	IARI, New Delhi

Table 1 continued

S. No	Botanical name	Accession no.	Code	Wild/ cultivated	Source of the material
26.	*V. reflexo-pilosa* Hayata var. *glabra* (Maréchal, Mascherpa et Stainier) Tateishi et Maxted	TCR20	TCR20glb	Wild	Harighat, Madhya Pradesh
27.	*V. trilobata* (L.) Verdcourt	BB2-2K	BB2_2Ktri	Wild	Raisen, Madhya Pradesh
28.	*V. trinervia* (Heyne ex Wight et Arn.) Tateishi et Maxted var. *bourneae* (Gamble) Tateishi et Maxted	TCR121	TCR121bour	Wild	Ninukuzhi, Kerala.
29.	*V. umbellata* (Thunb.) Ohwi et Ohashi	BB16-01A	BB16_01Aumb	Wild	Taradevi, Himachal Pradesh
30.	*V. umbellata* (Thunb.) Ohwi et Ohashi	Ricebean	Ricebean	Cultivated	Meghalaya
31.	*V. unguiculata* (L.) Walpers	Vyjayanthi	Vyjayanthi	Cultivated	KAU, Kerala

Table 2 The 10 selected deca-nucleotide primers along with their base sequences and characteristics of 152 RAPD amplification products studied in the analysis

S. no.	Primer	Base sequence (5′–3′)	No. of amplification products	Average no. amplification products per sample	Size range of amplicons (bp)
1.	OPA-13	CAGCACCCAC	15	6.488	250–1,050
2.	OPA-15	TTCCGAACCC	16	3.401	400–1,500
3.	OPA-16	AGCCAGCGAA	14	4.639	400–1,500
4.	OPB-02	TGATCCCTGG	14	2.406	400–1,500
5.	OPC-02	GTGAGGCGTC	13	4.279	200–1,031
6.	OPC-04	CCGCATCTAC	16	5.000	300–1,500
7.	OPC-08	TGGACCGGTG	17	6.197	150–1,250
8.	OPC-12	TGTCATCCCC	13	2.313	325–1,800
9.	OPD-13	GGGGTGACGA	19	7.250	200–1,500
10.	OPH-03	AGACGTCCAC	15	4.220	250–1,500

presence and 0 for absence). Genetic similarity (GS) between each pair of species was estimated using the method of Jaccard (1908): $GS = n_{xy}/n_t - n_z$, where n_{xy} is the number of bands common to accession A and B; n_t the total number of bands present in all samples and n_z the number of bands not present in both A and B but found in other samples. The similarity indices between the samples of *mungo–radiata* complex were also computed using the same formula. The amount of genetic variation within each population and species was quantified by determining number of polymorphic loci, percent polymorphic loci (p), observed number of alleles per locus (na) and effective number of alleles per locus (ne). Genetic diversity statistics were calculated after Nei (1987). Expected heterozygosity was calculated for all populations using the procedure of Lynch and Milligan (1994). At each polymorphic locus, the total allelic diversity was represented by Ht, which was partitioned into mean allelic diversity within populations (Hs) and the allelic diversity among populations (Dst). The proportion of total allelic diversity or genetic differentiation among populations (Gst) was calculated as the ratio Dst/Ht. F_{ST} values representing differentiation between populations were also calculated using Arlequin Version 3.01 software (Excoffier et al. 1992).

The Shannon–Weaver information index (Shannon and Weaver 1949) was calculated to measure the extent of diversity in each sample. The Wright's F-statistics, F_{CT}, F_{SC} and F_{ST} were also computed (Wright 1965). These are hierarchically related descriptors of the distribution of genetic variation within and among populations. The statistical

analyses were performed using POPGEN version 1.31 (http://www.ualberta.ca/~fyeh/fyeh).

An analysis of molecular variance (AMOVA) was also performed to partition the total genetic variation into that occurring within population, among population within groups and among groups using Arlequin Version 3.01 software (Excoffier et al. 1992). It was performed by grouping the populations conforming to species to two level of analysis, firstly, by including all 31 populations belonging to 14 *Vigna* species, secondly, by including only those species that belong to *mungo–radiata* complex. These two types of AMOVA were performed to resolve the differentiation of species within *mungo–radiata* complex.

The Jaccard's similarity matrix was utilized to construct a dendrogram using UPGMA procedure. Principal components analysis (PCA) was also performed to study the differences among the populations within a cluster. These analyses were conducted using NTSYS-pc software (Rohlf 1992).

Results

The 10 RAPD primers used (Table 2) generated 152 amplification products in the 152 individuals of 31 populations belonging to 14 *Vigna* species. The number of amplified fragments varied from 13 to 19 per primer. The pair-wise similarity values between species of *mungo–radiata* complex ranged from 0.081 to 0.910 with the average value of 0.339. The F_{ST} values (Table 3) between populations ranged from 0.084 (between accessions of *V. hainiana* i.e. BB-6-2K and TCR26) to 0.801 (between accessions of *V. mungo* and *V. radiata* var. *sublobata* TCR11).

The wild *Vigna* forms showed higher intra-specific variability in comparison to the cultivars (Tables 4, 5). Diversity parameters in the populations were indicators of the diversity prevalent in each of the species analyzed (Table 4). The number of polymorphic loci ranged from 15 (BBL69, *V. radiata* var. *setulosa*) to 99 (BB18-2K, *V. hainiana*) and the percent polymorphic loci varied from 9.87% (BBL69, *V. radiata* var. *setulosa*) to 65.13% (BB18-2K, *V. hainiana*). The estimates for observed and effective number of alleles per locus ranged from 1.09 (TCR66 of *V. radiata* var. *sublobata*) to 1.65 (BB18-2K of *V. hainiana*) and 1.06 (TCR66 of *V. radiata* var. *sublobata*) to 1.41 (BB18-2K of *V. hainiana*) respectively. The Nei's genetic diversity (h) estimates ranged from 0.04 (TCR66 of *V. radiata* var. *sublobata*) to 0.24 (BB18-2K, *V. hainiana*). The values for expected heterozygosity ranged from 0.07 (BBL69 of *V. radiata* var. *setulosa*) to 0.31 (BBL18-2K of *V. hainiana*). The Shannon-Weaver information index values varied from 0.09 (TCR66 of *V. radiata* var. *sublobata*) to 0.56 (BB18-2K, *V. hainiana*).

Inter-species variability was analyzed by computing the parameters, number of polymorphic loci (P), percent polymorphic loci, observed number of alleles per locus (na), effective number of alleles per locus (ne), Shannon-Weaver information Index (I), total diversity (Ht), diversity within accessions (Hs) and diversity between accessions, Dst (Table 5). Maximum number of polymorphic loci (136) and percent polymorphic loci (89.47) were observed for *V. hainiana* while, minimum number of polymorphic loci (30) and percent polymorphic loci (19.74) were observed for *V. radiata* var. *setulosa.* Observed number of alleles per locus (na) and effective number of alleles per locus (ne) ranged from 1.19 (*V. radiata* var. *setulosa*) to 1.71 (*V. hainiana*) and 1.13 (*V. radiata* var. *setulosa*) to 1.45 (*V. hainiana*) respectively. Shannon–Weaver information index (I) ranged from 0.1 (*V. radiata* var. *setulosa* and *V. reflexo-pilosa* var. *glabra*) to 0.4 (*V. hainiana*). Total diversity, which is partitioned into diversity within accessions and diversity between accessions varied from 0.07 (*V. radiata* var. *setulosa* and *V. reflexo-pilosa* var. *glabra*) to 0.26 (*V. hainiana*). The estimated within accession diversity ranged from 0.01 (*V. trilobata*) to 0.19 (*V. angularis*), whereas, between accession diversity ranged from 0.04 (*V. mungo*) to 0.12 (*V. trilobata*). The gene-flow among populations within species varied from 0 to 1.63 (*V. mungo*).

Wright Fixation indices (F_{SC}, F_{ST} and F_{CT}) were calculated to evaluate population subdivision and population substructure (Table 6). The computations for these indices were performed for all the populations and separately for *mungo–radiata* complex. For *mungo–radiata* relatives, F_{CT}, F_{SC} and F_{ST} estimates were 0.87, 0.43 and 0.56 respectively. Similarly for all *Vigna* species combined the fixation indices F_{CT}, F_{SC} and F_{ST} were 0.56, 0.42 and 0.25 respectively.

AMOVA (Table 7) indicated that 43.71% of the total variation was accounted by within population variation in comparison to 31.60% by among

Table 3 Pairwise comparison matrices of F_{ST} values of 31 populations computed using Arlequin Version 3.01 software (Excoffier et al. 1992)

	NG 2119 m	Mungo	PUSA 105rad	UPM 92rad	TCR 27 hai	TCR 29 hai	TCR 24hai	TCR 26hai	BB6-2Khai	BB18-2Khai	BBD7-01Bhai	BB25-01Bhai	BBL55-2Kmsil	BBL60-2Kmsil	BBL40-2Kmsil
Mungo	0.369														
PUSA105rad	0.5	0.649													
UPM92rad	0.624	0.741	0.246												
TCR27hai	0.561	0.673	0.518	0.581											
TCR29hai	0.491	0.593	0.470	0.516	0.174										
TCR24hai	0.546	0.643	0.488	0.568	0.436	0.251									
TCR26hai	0.567	0.688	0.525	0.591	0.516	0.410	0.167								
BB6-2Khai	0.477	0.616	0.442	0.517	0.453	0.318	0.184	0.084							
BB18-2Khai	0.419	0.534	0.395	0.454	0.385	0.323	0.200	0.188	0.111						
BBD701Bhai	0.621	0.707	0.576	0.624	0.533	0.413	0.377	0.485	0.418	0.306					
BB25-01Bhai	0.563	0.646	0.548	0.612	0.534	0.457	0.492	0.541	0.463	0.384	0.499				
BBL55-2Ksil	0.609	0.729	0.626	0.671	0.671	0.591	0.634	0.654	0.586	0.466	0.664	0.484			
BBL60-2Ksil	0.549	0.640	0.559	0.598	0.574	0.517	0.531	0.572	0.508	0.385	0.568	0.405	0.184		
BBL40-2Ksil	0.587	0.715	0.580	0.653	0.624	0.549	0.552	0.576	0.503	0.383	0.633	0.494	0.386	0.188	
BBL50-2Ksil	0.606	0.668	0.551	0.619	0.614	0.563	0.562	0.599	0.544	0.443	0.609	0.473	0.332	0.270	0.321
BBL83-2Ksil	0.553	0.635	0.520	0.580	0.565	0.508	0.526	0.576	0.489	0.396	0.585	0.435	0.415	0.341	0.381
TCR11sub	0.713	0.801	0.630	0.703	0.704	0.655	0.693	0.702	0.631	0.533	0.696	0.595	0.716	0.649	0.707
TCR62sub	0.654	0.733	0.653	0.697	0.655	0.574	0.574	0.615	0.551	0.455	0.652	0.598	0.644	0.521	0.616
BBL64-2Ksub	0.687	0.775	0.701	0.753	0.703	0.620	0.656	0.689	0.629	0.509	0.717	0.613	0.721	0.631	0.676
TCR-66s	0.602	0.770	0.638	0.712	0.653	0.557	0.558	0.590	0.471	0.384	0.655	0.556	0.671	0.534	0.604
BBL72-2Ks	0.715	0.812	0.710	0.761	0.724	0.677	0.705	0.713	0.653	0.550	0.755	0.667	0.726	0.637	0.670
TCR20glbr	0.627	0.710	0.643	0.702	0.629	0.561	0.598	0.647	0.584	0.495	0.665	0.596	0.672	0.568	0.639
TCR69dalz	0.579	0.660	0.585	0.628	0.584	0.519	0.563	0.617	0.543	0.473	0.620	0.580	0.626	0.531	0.579
BB2-2Ktri	0.551	0.599	0.535	0.586	0.550	0.487	0.498	0.553	0.487	0.415	0.566	0.538	0.601	0.513	0.584
TCR121brn	0.568	0.648	0.568	0.610	0.548	0.499	0.512	0.575	0.511	0.412	0.580	0.574	0.627	0.515	0.586
BBL79-2Kmn	0.592	0.662	0.582	0.626	0.590	0.542	0.557	0.592	0.534	0.459	0.631	0.608	0.670	0.579	0.629
BB16-2Kumb	0.620	0.675	0.573	0.629	0.616	0.553	0.581	0.631	0.555	0.494	0.640	0.594	0.649	0.556	0.624
umbel	0.625	0.699	0.610	0.653	0.569	0.520	0.574	0.614	0.554	0.430	0.596	0.579	0.667	0.524	0.612
ungic	0.582	0.640	0.527	0.570	0.542	0.500	0.515	0.579	0.521	0.436	0.559	0.530	0.631	0.533	0.608
vangul	0.507	0.586	0.462	0.514	0.506	0.433	0.429	0.488	0.413	0.307	0.500	0.444	0.547	0.488	0.524

Table 3 continued

	BBL50-2Kmsil	BBL83-2Kmsil	TCR11sub	TCR 62sub	BBL64-2Ksub	TCR-66s	BBL72-2Ks	TCR 20glbr	TCR69 dalz	BB2-2Ktrb	TCR 121brn	BBL79-2Kmn	BB16-2 Kumb	umbel	ungic	Angul
Mungo																
PUSA105rad																
UPM92rad																
TCR27hai																
TCR29hai																
TCR24hai																
TCR26hai																
BB6-2Khai																
BB18-2Khai																
BBD701Bhai																
BB25-01Bhai																
BBL55-2Ksil																
BBL60-2Ksil																
BBL40-2Ksil																
BBL50-2Ksil																
BBL83-2Ksil	0.310															
TCR11sub	0.650	0.543														
TCR62sub	0.565	0.565	0.735													
BBL64-2Ksub	0.668	0.655	0.774	0.644												
TCR-66s	0.587	0.592	0.769	0.606	0.496											
BBL72-2Ks	0.673	0.664	0.783	0.686	0.656	0.608										
TCR20glbr	0.601	0.594	0.760	0.674	0.736	0.666	0.723									
TCR69dalz	0.573	0.529	0.673	0.576	0.613	0.539	0.613	0.482								
BB2-2Ktri	0.526	0.501	0.616	0.500	0.600	0.518	0.647	0.471	0.454							
TCR121brn	0.551	0.528	0.641	0.544	0.631	0.533	0.642	0.543	0.469	0.379						
BBL79-2Kmn	0.596	0.586	0.668	0.602	0.682	0.562	0.685	0.514	0.509	0.341	0.409					
BB16-2Kumb	0.589	0.555	0.670	0.612	0.705	0.622	0.721	0.553	0.509	0.436	0.508	0.391				
umbel	0.595	0.579	0.696	0.604	0.707	0.626	0.704	0.613	0.535	0.465	0.511	0.493	0.538			
ungic	0.566	0.556	0.602	0.526	0.601	0.523	0.604	0.514	0.472	0.350	0.361	0.440	0.478	0.458		
vangul	0.499	0.470	0.583	0.506	0.574	0.453	0.558	0.446	0.442	0.417	0.422	0.449	0.462	0.450	0.367	

population variation within the species, and among species variation accounted for 24.71% of total variation. Similarly, for *mungo–radiata* complex, of the total genetic diversity, 40.42% was attributed to individuals within populations, 30.95% to populations within species and 28.63% to amongst species.

The UPGMA tree constructed on the basis of Jaccard's similarity matrix by incorporating *V. unguiculata* as an outgroup (Fig. 1) classified the populations representing six species of mungo–radiata complex into two groups (I and II). Out of six species, group I included all populations belonging to *V. radiata*, *V. mungo*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa* and *V. mungo* var. *silvestris*, whereas, group II incorporated all the populations belonging to *V. hainiana*. The PCA analysis (Fig. 2) was performed for *mungo–radiata* complex. The six species segregated into six groups, with all populations of a species forming a distinct single group. The first three most informative principal components accounted for 14.82%, 10.82% and 9.20% of the total variation and 22 components are required to explain the total variation.

Discussion

The knowledge of population substructure, genetic diversity and phylogenetic relationships between wild forms and cultigens of *Vigna* is essential for devising strategies for efficient genetic diversity maintenance and utilization. The environmental challenges faced by wild and cultivated species, today, underline the innate importance of measurement and assessment of genetic variability. The loss of genetic diversity is the major threat for the maintenance and adaptive potential of species. Moreover, accurate identification of genetic relatedness between cultivars and varieties is a pre-requisite for plant breeding programmes. In the present study, extensive genetic variability, high differentiation among species and strong subdivision among populations of the species were observed. High polymorphism observed for amplicons generated with each primer can be attributed to the fact that 14 distinct and diverse species of *Vigna* were analyzed in the present study. In fact, earlier only 3% bands were reported as common in 23 accessions belonging to subgenus *Ceratotropis* (Kaga et al. 1996).

Of the species analyzed, higher polymorphism was observed for the wild species in comparison to the cultivated ones except for the wild species where sample size is very small due to their limited occurrence in the wild habitats (Tables 4, 5). This is in contrast to the high genetic similarity reported earlier among the cultivars of *V. radiata* using RAPDs (Lakhanpaul et al. 2000). Perusal of the estimates of genetic diversity statistics among the populations representing various species indicated high levels of population differentiation. Averaged over all loci, the total genetic diversity is moderate and high level of variability is found between species. However, total diversity (Ht), within accession diversity (Hs) and between accession diversity (Dst) observed were higher than earlier studies based on isozyme electrophoresis, where values for total diversity is less than 0.2 for *V. vexillata* (Sonnante et al. 1997) and *V. luteola* and *V. marina* (Sonnante et al. 1998) and 0.3 for *V. unguiculata* (Pasquet 1999). In these reports, the within accession diversity reported was less than 0.01 (Sonnante et al. 1997; Pasquet 1999). Higher diversity and differentiation reported in the present study could be due to presence of greater diversity in wild populations of the species and higher polymorphism observed for the RAPD markers in comparison to isozymes and also due to the fact that larger number of polymorphic markers was available with RAPDs (Williams et al. 1990). On the other hand, protein/isozymes profiles reveal only a fraction of the genetic changes in the coding regions of the genome.

Considerable gene-flow was detected between the populations of *V. mungo*, *V. mungo* var. *silvestris*, *V. radiata*, *V. radiata* var. *sublobata*, *V. hainiana* and *V. umbellata*. This indicates occurrence of intercrossing between wild populations of these species, as these populations occur in contiguous areas and mostly their geographic areas of occurrence overlap (Table 1). Minimum values for F_{ST} were observed between populations of *V. hainiana* indicating that these populations are least differentiated, whereas, within a group maximum values of F_{ST} was recorded for populations of *V. radiata* var. *sublobata* indicating that this group comprises divergent populations.

Fixation index is defined as increased homozygosity resulting from inbreeding. Wright's F statistics could be negative or positive indicating a deficiency or excess of homozygotes in relation to a random

Table 4 Genic diversity paramers for the *Vigna* populations based on RAPD polymorphism

Population code	Sample size	No. of polymorphic loci	% Polymorphic loci (p)	Observed number of alleles per locus (na)	Effective number of alleles per locus (ne)	Nei's genic diversity (h)	Shannon–Weaver information index (I)	Exp. heterozygosity
mungo	5	24	15.79	1.15 ± 0.36	1.09 ± 0.25	0.56 ± 0.14	0.08 ± 0.20	0.09 ± 0.016
NG2119mun	5	48	31.58	1.13 ± 0.44	1.20 ± 0.34	0.11 ± 0.18	0.17 ± 0.26	0.09 ± 0.016
BBL83_2Kmsil	5	27	17.76	1.40 ± 0.48	1.25 ± 0.37	0.14 ± 0.19	0.21 ± 0.28	0.18 ± 0.019
BBL55_2Kmsil	4	35	23.02	1.23 ± 0.42	1.13 ± 0.29	0.08 ± 0.16	0.12 ± 0.23	0.12 ± 0.018
BBL60_2Kmsil	5	63	41.45	1.41 ± 0.49	1.26 ± 0.36	0.15 ± 0.19	0.22 ± 0.28	0.09 ± 0.016
BBL40_2Kmsil	4	47	30.92	1.30 ± 0.46	1.22 ± 036	0.12 ± 0.19	0.18 ± 0.28	0.16 ± 0.020
BBL50_2Kmsil	5	57	37.50	1.37 ± 0.48	1.23 ± 0.35	0.13 ± 0.19	0.19 ± 0.27	0.17 ± 0.019
Pusa105rad	5	54	35.53	1.35 ± 0.48	1.32 ± 0.36	0.12 ± 0.19	0.19 ± 0.27	0.16 ± 0.018
UPM 92rad	5	34	22.37	1.22 ± 0.41	1.15 ± 0.31	0.08 ± 0.17	0.12 ± 0.24	0.11 ± 0.017
TCR62sub	5	41	26.97	1.17 ± 0.38	1.16 ± 0.31	0.09 ± 0.17	0.14 ± 0.24	0.12 ± 0.017
TCR11sub	5	27	17.76	1.26 ± 0.44	1.13 ± 0.30	0.07 ± 0.16	0.10 ± 0.23	0.09 ± 0.016
BBL64_2Ksub	5	26	17.11	1.17 ± 0.37	1.11 ± 0.28	0.06 ± 0.15	0.09 ± 0.22	0.09 ± 0.016
BBL69s	2	15	9.87	1.09 ± 0.29	1.06 ± 0.21	0.04 ± 0.12	0.56 ± 0.18	0.07 ± 0.018
BBL72_2Ks	5	30	19.74	1.19 ± 0.39	1.13 ± 0.30	0.07 ± 0.16	0.10 ± 0.23	0.09 ± 0.016
BB25_01Bhai	5	66	43.42	1.43 ± 0.49	1.26 ± 0.38	0.15 ± 0.19	0.22 ± 0.28	0.18 ± 0.018
BB6_2Khai	5	72	47.37	1.47 ± 0.50	1.30 ± 0.39	0.17 ± 0.20	0.25 ± 0.29	0.21 ± 0.019
BB7_01Bhai	5	52	34.21	1.34 ± 0.50	1.19 ± 0.33	0.11 ± 0.17	0.17 ± 0.25	0.15 ± 0.017
BBL18_2Khai	6	99	65.13	1.65 ± 0.47	1.41 ± 0.37	0.24 ± 0.20	0.35 ± 0.28	0.31 ± 0.020
TCR24hai	4	51	33.55	1.33 ± 0.47	1.20 ± 0.32	0.11 ± 0.18	0.17 ± 0.26	0.18 ± 0.020
TCR26hai	5	57	37.50	1.37 ± 0.48	1.21 ± 0.34	0.12 ± 0.18	0.19 ± 0.26	0.17 ± 0.019
TCR27hai	6	56	36.84	1.36 ± 0.48	1.21 ± 0.34	0.12 ± 0.18	0.18 ± 0.26	0.15 ± 0.017
TCR29hai	7	63	41.45	1.41 ± 0.49	1.24 ± 0.34	0.14 ± 0.19	0.21 ± 0.27	0.19 ± 0.019
Ricebean umb	4	48	31.58	1.32 ± 0.46	1.21 ± 0.35	0.12 ± 0.19	0.17 ± 0.27	0.17 ± 0.020
BB16_01Aumb	5	50	32.89	1.32 ± 0.47	1.20 ± 0.34	0.12 ± 0.18	0.17 ± 0.26	0.16 ± 0.019
BBL79_2Kmin	5	57	37.50	1.37 ± 0.48	1.18 ± 0.29	0.11 ± 0.16	0.18 ± 0.24	0.18 ± 0.019
TCR121trinervia	5	63	41.45	1.41 ± 0.49	1.26 ± 0.37	0.15 ± 0.19	0.22 ± 0.28	0.19 ± 0.019
TCR20glb	3	31	20.39	1.20 ± 0.40	1.13 ± 0.29	0.07 ± 0.16	0.11 ± 0.23	0.12 ± 0.019
BBL2_2Ktri	5	60	39.47	1.39 ± 0.49	1.23 ± 0.33	0.13 ± 0.18	0.20 ± 0.27	0.19 ± 0.020
BBL69_2Kdalz	6	60	39.47	1.39 ± 0.49	1.23 ± 0.35	0.13 ± 0.19	0.20 ± 0.27	0.16 ± 0.017
unguic	6	69	45.39	1.45 ± 0.49	1.26 ± 0.36	0.15 ± 0.19	0.23 ± 0.27	0.20 ± 0.019
angul	5	75	49.34	1.49 ± 0.50	1.34 ± 0.39	0.19 ± 0.21	0.28 ± 0.30	0.25 ± 0.021

mating population. F equals to zero, when the population is in Hardy–Weinberg equilibrium, while when all genotypes are homozygous, F equals 1. In the present study, high estimates of fixation index indicated a predominantly autogamous behavior of the wild species. Hartl (1987) suggested that the range 0–0.05 indicates little differentiation, 0.05–0.15 moderate, 0.15–0.25 large differentiation and above 0.25 indicates very large differentiation. The high values for Fct observed here (which is equivalent to Wright's Fst), indicates strong subdivision of populations representing a species and high genetic differentiation among the species. A comparison of the Fct values for the *mungo–radiata* complex (0.87) and all the *Vigna* species together (0.25) indicates existence of very large genetic differentiation among the designated species and lower taxonomic levels in this complex. The partitioning of molecular variance with AMOVA revealed that substantial variation exists among the species and populations indicating

Table 5 Estimates of genic divergence statistics among 14 *Vigna* species based on polymorphic RAPD markers

Gr. No.	Group	Sample size	No. of polymorphic loci (P)	% Polymorphic loci	Observed no. of alleles per locus (na)	Effective no. of alleles/ locus (ne)	Shannon–Weaver information index (I)	Gene differentiation (Gst)	Gene flow (Nm)	Total diversity (Ht)	Diversity within accession (Hs)	Diversity between accession (Dst)
1.	*V. angularis*	5	75	49.34	1.45 ± 0.50	1.34 ± 0.39	0.28 ± 0.30	0.00	–	0.19 ± 0.04	0.19 ± 0.04	–
2.	*V. trinervia* var. *bourneae*	5	63	41.45	1.41 ± 0.49	1.26 ± 0.37	0.22 ± 0.28	0.00	–	0.15 ± 0.03	0.15 ± 0.02	–
3.	*V. dalzelliana*	6	60	39.47	1.39 ± 0.49	1.23 ± 0.35	0.20 ± 0.27	0.00	–	0.13 ± 0.03	0.13 ± 0.03	–
4.	*V. reflexo-pilosa* var. *glabra*	3	31	20.39	1.20 ± 0.40	1.13 ± 0.29	0.11 ± 0.23	0.00	–	0.07 ± 0.02	0.07 ± 0.02	–
5.	*V. hainiana*	43	136	89.47	1.89 ± 0.30	1.42 ± 0.32	0.40 ± 0.22	0.42	0.67	0.26 ± 0.02	0.15 ± 0.01	0.09
6.	*V. minima*	5	57	37.50	1.37 ± 0.48	1.18 ± 0.29	0.18 ± 0.24	0.00		0.11 ± 0.02	0.11 ± 0.02	–
8.	*V. mungo*	10	67	44.08	1.44 ± 0.49	1.23 ± 0.33	0.21 ± 0.26	0.23	1.63	0.14 ± 0.03	0.10 ± 0.02	0.04
9.	*V. m. silvestris*	23	116	76.32	1.76 ± 0.42	1.35 ± 0.33	0.34 ± 0.25	0.41	0.70	0.21 ± 0.03	0.12 ± 0.01	0.09
7.	*V. r. sulosa*	5	30	19.74	1.19 ± 0.39	1.13 ± 0.30	0.10 ± 0.23	0.00	–	0.07 ± 0.02	0.07 ± 0.02	–
10.	*V. r. sublobata*	17	108	71.05	1.71 ± 0.45	1.45 ± 0.37	0.39 ± 0.27	0.73	0.18	0.25 ± 0.03	0.16 ± 0.01	0.09
11.	*V. radiata*	10	66	43.42	1.43 ± 0.49	1.43 ± 0.34	0.21 ± 0.27	0.39	0.77	0.14 ± 0.03	0.08 ± 0.01	0.06
12.	*V. trilobata*	5	60	39.47	1.39 ± 0.49	1.23 ± 0.33	0.20 ± 0.27	0.00	–	0.13 ± 0.03	0.01 ± 0.03	0.12
13.	*V. umbellata*	09	95	62.50	1.62 ± 0.48	1.36 ± 0.37	0.31 ± 0.28	0.43	0.65	0.21 ± 0.03	0.12 ± 0.03	0.09
14.	*V. unguiculata*	6	69	49.39	1.45 ± 0.49	1.26 ± 0.36	0.23 ± 0.27	0.00	–	0.15 ± 0.03	0.15 ± 0.03	–

Table 6 Wright's fixation indices for the 14 *Vigna* species and *mungo–radiata* complex based on RAPD markers

S. No.	Population differentiation indices	Combined for all *Vigna* species	For mungo–radiata relatives
1.	Fsc	0.42	0.43
2.	Fst	0.56	0.56
3.	Fct	0.25	0.87

that greater number of population samples should be sampled and studied to delineate the species identities and boundaries in this species complex. It could be seen that the partitioning of variation is similar in magnitude in both *mungo–radiata* complex as well as when all 14 *Vigna* species are compared together.

UPGMA tree based on RAPD profiles indicated that *V. mungo*, *V. radiata*, *V. mungo* var. *silvestris*, *V. radiata* var. *sublobata* and *V. radiata* var. *setulosa* are distinct taxonomic groups. However, as these species were placed in the same cluster in the dendrogram, their origin from a common ancestor is evident. In addition, cultivated forms are grouped close to their proposed wild forms. Though, Kaga et al. (1996) reported similar grouping in their study of subgenus *Ceratotropis*, more variation between

Table 7 Analysis of molecular variance (AMOVA) for 152 individuals representing 14 *Vigna* species and partitioning of molecular variance in *mungo–radiata* species complex

S. No.	Source of variation	Combined for all 14 *Vigna* species			For *mungo–radiata* relatives		
		DF	Variance components	% Of variation	DF	Variance components	% Of variation
1.	Among groups	15	7.47	24.70	5	8.76	28.63
2.	Among populations within groups	18	9.55	31.59	16	9.47	30.95
3.	Within populations	138	13.21	43.71	86	12.37	40.42
	Total	171	30.23		107	30.60	

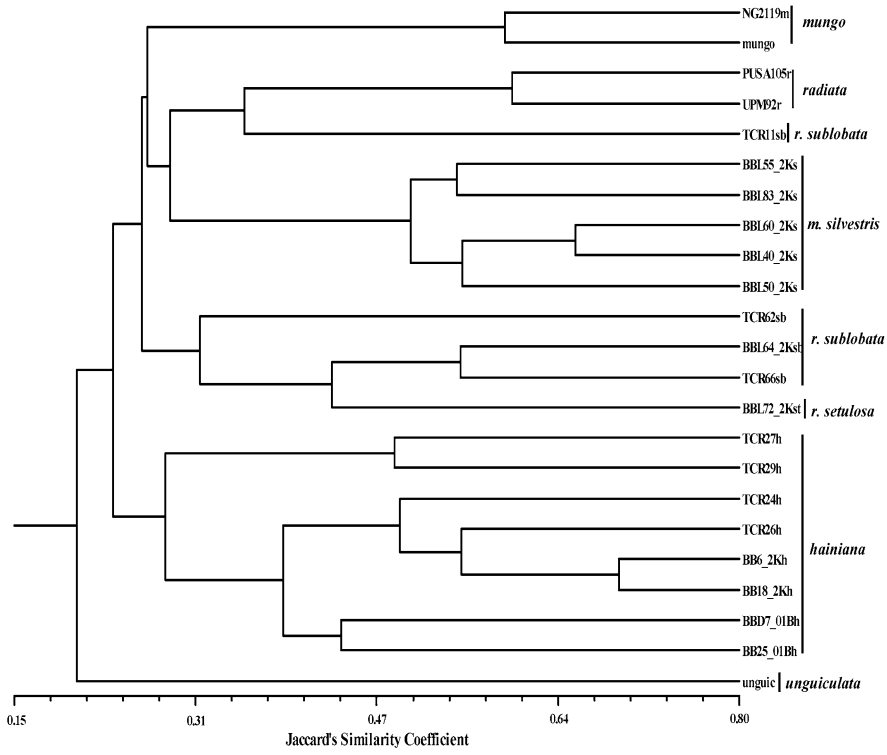

Fig. 1 UPGMA tree based on RAPD amplicons illustrating relationships among the six *Vigna* species belonging to the *mungo-radiata* complex using *V. unguiculata* as an outgroup

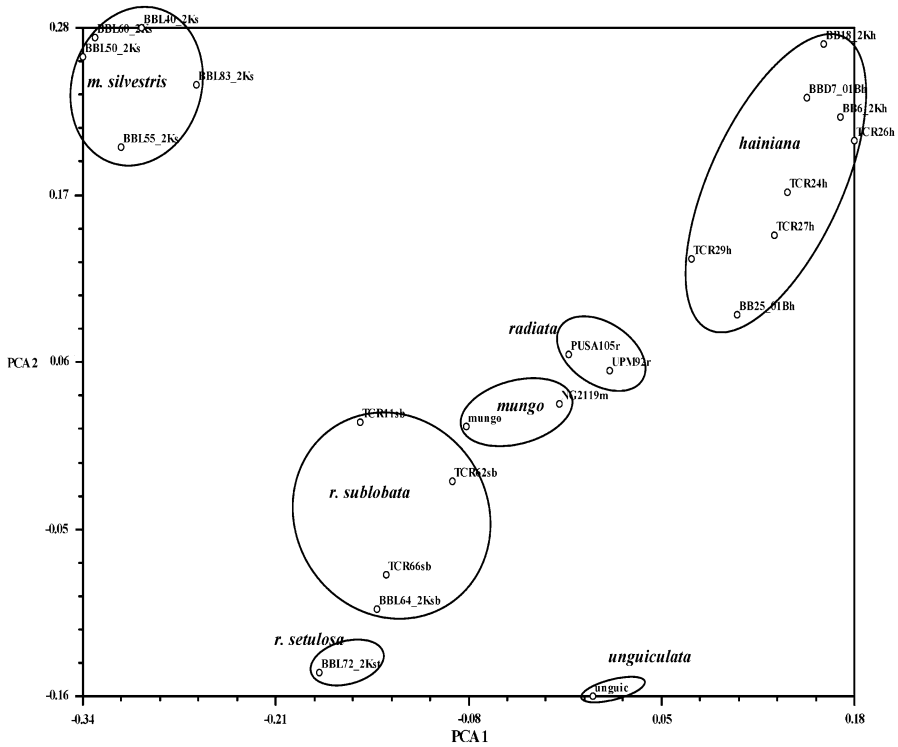

Fig. 2 Principal components analysis of RAPD profiles to resolve relationships among the *Vigna* species belonging to the *mungo–radiata* complex

cultivated and wild forms was found in *V. radiata* than in *V. mungo*, in the present study. Therefore, wild forms of *V. radiata* have differentiated greatly since domestication and some of them retain only partial affinity with the related species *V. mungo* (Miyazaki 1982). Clustering of all the accessions of *V. hainiana* together suggests it to be a distinct taxa and its close and equidistant grouping also appeared to support the view that *V. hainiana* might be the common putative progenitor of wild relatives of both *V. mungo* and *V. radiata* (Babu et al. 1985). However, elaborate analysis of diverse populations is required to further confirm whether *V. hainiana* could be considered as the putative progenitor of green gram and black gram and whether the closest wild relative of green gram, namely, *V. radiata* var. *sublobata* and *V. radiata* var. *setulosa* also have their origin from *V. hainiana*.

To conclude, the evidences suggest that: (1) the wild species, *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris* have high similarity to their respective cultivated forms. (2) *V. hainiana*, *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa* and *V. mungo* var. *silvestris* are distinct taxa. (3) *V. hainiana* is equidistant to both *V. radiata* and *V. mungo*. (4) *V. hainiana* is more primitive compared to *V. radiata* var. *sublobata*, *V. radiata* var. *setulosa* and *V. mungo* var. *silvestris*. Hence, *V. hainiana* could be the common pivotal progenitor species of both *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris* from which the cultivated mung (*V. radiata*) and urd (*V. mungo*) have evolved.

Acknowledgements The facilities provided by Director, National Bureau of Plant Genetic Resources for conducting this work is gratefully acknowledged. RV gratefully acknowledges receipt of research fellowship for her Ph.D. work from Council of Scientific and Industrial Research, Delhi.

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