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PHYLOGENY OF *VIGNA* SPECIES AS INDICATED BY SEED PROTEIN AND ISOZYME ELECTROPHORESIS

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The evolutionary relationships between cultivated and wild species of Asiatic Vigna *species were investigated through biochemical parameters utilizing isozyme variation patterns of peroxidase, catalase and seed protein polymorphism studied through polyacrylamide gel electrophoresis. The results of above investigations are presented in this paper. The studies provide strong evidence in elucidation of phylogenetic relationships. The results indicated that* mung *and* urid *beans are two distinct species and they have independently evolved from wild progenitors* V. radiata *var.* sublobata *and* V. mungo *var.* silvestris, *respectively, which show remarkable similarities in isozyme profiles particularly in number of bands, position and Rf values. The wild putative forms occurring in natural habitats belonging to* V. umbellata, V. aconitifolia *and* V. trilobata *also appear to be quite distinct. They exhibited fewer bands. Close affinity could not be discovered as revealed by protein and isozyme polymorphism.*

Seed protein gel electrophoresis techniques have been used to establish the origin of some taxa in plants (Gallez and Gottlieb, 1982) and in animals (Sekiguchi and Sugita, 1980). The electrophoretic analysis involving both seed proteins and isozymes provide useful evidences in the study of phenetic variation at the lowest taxonomic level in terms of intensity of common bands and presence or absence of other bands or their displacement. Isozyme polymorphism has been used for legume and bean systematics where electrophoretic mobility values of formic and glutamic acid dehydrogenases were determined in legume species from different tribes, including beans (Thurman *et al.*, 1967). On the basis of

electrophoretic patterns of seed proteins, the *Phaseolus* species of American and Asiatic origin were clearly differentiated and grouped separately (Derbyshire *et al.*, 1976). The isozyme electrophoretic patterns in bean species studied by Bassiri and Adams (1978 a) showed that most of them were characterized by unique isozyme spectrum. The origin and evolutionary relationships between several predominantly cultivated taxa such as mung bean (*V. radiata* (L.) Wilczek), urid bean (*V. mungo* (L.) Hepper), rice bean (*V. umbellata* (Thunb.) Ohwi and Ohashi), moth bean (*V. aconitifolia* (Jacq.) Marechal) and *V. trilobata* (Ait.) with their closely related wild species particularly *V. radiata* var. *sublobata* (Roxb.) Verdc. and *V. mungo* var. *silvestris* Lukoki, Marechal and Otoul (1980) have remained a subject of intensive research. The present investigations were aimed to have comparative study of isoenzymes particularly peroxidase, catalase and total seed proteins among cultivated Asiatic *Vigna* species and their related wild taxa.

MATERIALS AND METHODS

The germplasm collections of cultivated and wild species of *Vigna*, being maintained by NBPGR and also regenerated from time to time in the field and greenhouse, were used for this study. The wild species and their diverse morphotypic forms were grown in the green house during the rainy season (July-November), 1988 and 1989 and fresh materials were used for present studies. The details of the species, their accessions, source of collection etc. are presented in Table 1.

Electrophoretic studies on seed proteins were conducted on samples of seeds obtained from the 1988/1989 raised crops. Four accessions each in *V. radiata* var. *sublobata, V. mungo* var. *silvestris, V. umbellata* var. *major* and var. *rumbaiya* and *V. trilobata* were utilised. The typical cultivars of mung, urid, rice bean and moth bean were also included. A complete schematic representation of the protein profiles (banding patterns) have been depicted in Fig. 1. Similarity indices among the protein profiles of *Vigna* genotypes have been calculated and shown in Table 3. Isozyme studies were conducted on the fresh leaves obtained from 5 months old plants. Three other wild species; *V. vexillata, V. dalzaliana* and *V. aconitifolia* were included in addition to above mentioned species. The relative mobility of two isoenzymes, peroxidase and catalase is indicated in Table 4.

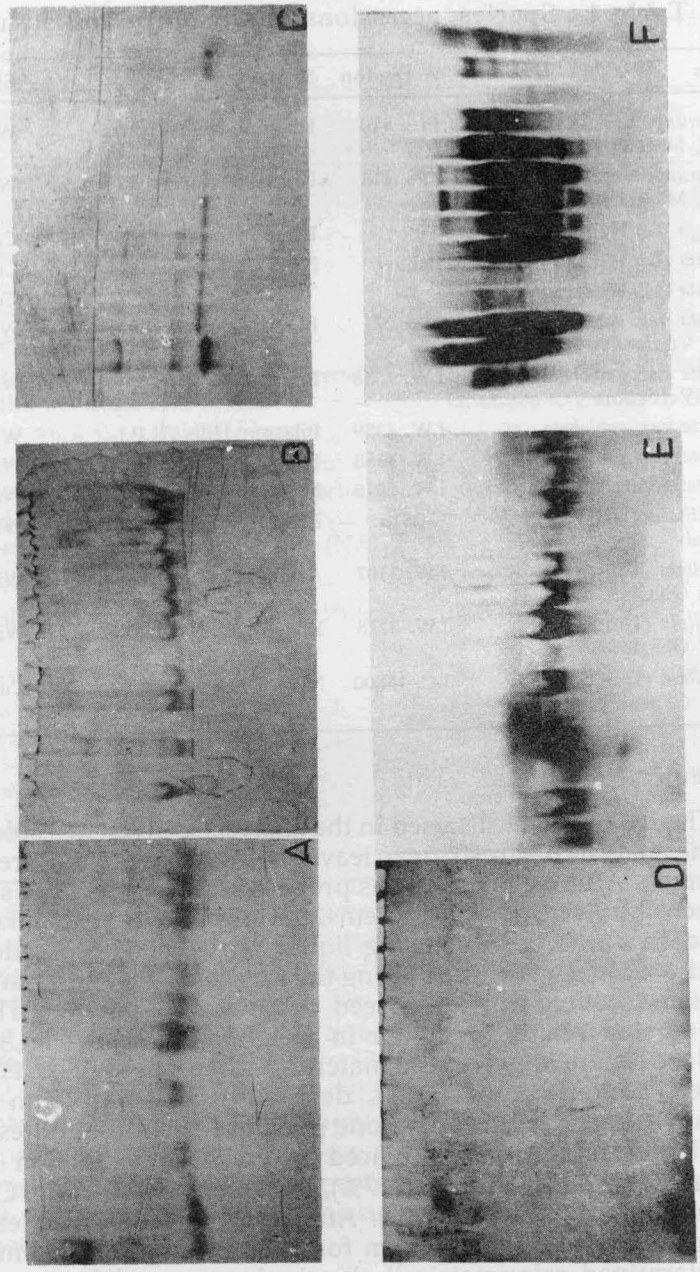

Fig. 1 : Schematic representation of banding patterns in *Vigna* species complex

Table 1 : Species, accessions, their source and status

Species	Accession	Source	Status
Vigna mungo var. *silvestris* Lukoki, Marechal and Otoul	Pl x 410	Khopoli, Maharashtra	Wild
Vigna mungo var. *silvestris* Lukoki, Marechal and Otoul	Pl x 416	Chipulin, Karnataka	Wild
V. mungo (L.) Hepper	T_9	Uttar Pradesh	Cultivated
V. radiata (L.) Wilczek	Pallavi	NBPGR, New Delhi	Cultivated
V. radiata (L.) Wilczek	—	—	Cultivated
V. radiata var. *sublobata* (Roxb.) Verdcourt	Pl x 271	Bhowali, U.P. Hills	Wild
V. radiata var. *sublobata* (Roxb.) Verdcourt	I.W. 4358	Bilaspur Hills (H.P.)	Wild
V. radiata var. *sublobata*	I.W. 4359	Bilaspur Hills (H.P.)	Wild
V. radiata var. *sublobata*	I.W. 4360	Bilaspur Hills (H.P.)	Wild
V. trilobata (Ait) Jacq.	I.W. 2549	NBPGR, New Delhi	Wild
V. aconitifolia (Jacq,) Marechal	C-2515	Rajendranagar, Hyderabad	Wild
V. umbellata (Thunb.) Ohwi & Ohashi	I.W. 3107	Arbila peak, Meghalaya	Wild
V. umbellata (Thunb.) Ohwi & Ohashi	I.W. 3178	Khasi hills, Meghalaya	Wild
V. umbellata (Thunb.) Ohwi & Ohashi	I.C. 16800	NBPGR, New Delhi	Cultivated

Biochemical methods

The list of chemicals used in the analysis is given in Table 2. For isozyme analysis the fresh green leaves of 5 months old mature plants were taken. Acetone powder was prepared from each of the sample. For electrophoresis of seed proteins, the fresh seeds were taken and crushed to a fine powder using liquid nitrogen. The powder was washed with acetone while putting the extract on the funnel and care was taken to avoid mixing of seed coat with the powder. The fine powder being relatively lighter in weight than seed coat, it was possible to decant only the seed material without the seed coats. Thus, prepared acetone powder was defatted using petroleum ether. Protein was extracted from acetone powder for 10-15 minutes using a prechilled mortar pestle placed in ice. The extraction buffer contained Tris (0.1 M), Sucrose (17%), ascorbic acid (0.1%) and Cystine hydrochloride (0.1%) at pH 7.4. After crushing, the samples were centrifuged at 4°C at 17,000 rpm for 15 min. Protein concentration was determined colorimetrically by the Lowry modification of the Folin method (Lowry *et al.*, 1951) using the supernatant. Vertical migration towards the anode on 7.5 per cent polyacrylamide was then performed using Pharmacia Slab Gel Electrophoresis Apparatus

Table 2 : List of chemicals used, their quality brand name and source

S.No.	Name of Chemical/s	Quality Brand Name (Product/Source)
(1)	Sodium Thiosulfate, Disodium Hydrogen Phosphate, Sodium Citrate and Urea	E MERC, Bombay, India
(2)	Potassium Iodide, Sodium Dihydrogen Phosphate and Acetic Acid	Glaxo, Bombay, India
(3)	Fast Blue RR Salt, Hydrogen Peroxide, Sodium Acetate and Iodine	BDH Laboratory Chemicals, England, U.K.
(4)	Nitro Blue Tetrazolium (NBT), Dithiothesitol (DTT), Phenyl Methyl Sulfonate (PMS), Hypoxanthine, aspartic acid, Pyridoxal Phosphate and Nicotinamide Adenine Dinucleotide (NAD)	SRL, Bombay, India
(5)	Acetone	Qualigens, India
(6)	Trishydroxyl Methyl Aminomethane (TRIS) and Calcium Chloride	Spectrochem, India
(7)	Ethanol	Bengal Chemicals, India
(8)	Fast Blue BB Salt, D.L. Malate, 3- (4,5-Dimethyethiazol-2-yl) -2.5- Diphenyl Tetrazolium Bromide	Sigma Chemicals Co. U.S.A.
(9)	Starch	Indian Drugs and Pharmaceuticals Ltd. India

GE 2/4LS allowing the simultaneous electrophoresis of 14 samples for a gel of 0.4 m thickness. Reservoir buffer contained Tris (0.025 M) and glycine (0.133 M) at pH 8.3 Electrophoresis was performed at 70V for 4-5 hours. The staining for different isozymes was done following the procedures of Vallejos (1983). Proteins were stained using the procedure described by Davis (1964), with 25 per cent methanol and 10 per cent acetic acid (glacial). The gels were examined and photographed while on a light box. The similarity between various protein profiles and isozyme patterns were expressed in the percentage similarity index. The percentage similarity (Whiteney *et al.*, 1968) was computed for the various species/genotypes of the *Vigna* species.

$$\text{Percentage similarity} = \frac{\text{No. of pairs of similar bands}}{\text{No. of different bands + No. of pairs of similar bands}} \times 100$$

RESULTS AND DISCUSSION

Several cultivated and wild species of *Vigna* and their polymorphic forms studied in the present investigations, produced very characteristic protein profiles. The total number of visible bands varied from 16 to 30. The data in Table 3 indicated the number of bands and relative mobility of two isoenzymes, peroxidase and catalase. Rf values of differential bands among the various genotypes indicated that the fastest migrating band had a Rf value of 0.57. In case of *V. umbellata, V. vexillata, V. dalzaliana* and other wild *Vigna* species no specific enzymatic activity was exhibited for peroxidase, and catalase enzymes. The variation patterns among the closely related groups were determined on the basis of electrophoretic patterns of both isozyme profiles as well as protein polymorphism.

Table 3 : Similarity indices among the profiles of *Vigna* spp. genotypes

	V. radiata var. *sub-lobata* PI x 271	*V. radiata* var. *sub-lobata* IW4358	*V. radiata* var. *sub-lobata* IW4359	*V. radiata* var. *sub-lobata* IW4360	*V. trilobata* IW 2549	*V. mungo* var. *syl-vestris* PI x 410	*V. mungo* var. *syl-vestris* PI x 416	*V. umbe-llata* IW 3178
V. radiata PIMS (Pallavi)	55.5	50	51.4	48.5	40	65.6	58.8	34.2
V. mungo T-9 (urid)	47.5	—	—	—	37.2	60	58.3	35.0
V. mungo var. silvestris PI x 410	—	—	—	—	—	—	70.9	—

The results of the biochemical studies showed that considerable similarities exist between the cultivated species mung bean (*V. radiata* (L.) Wilczek) and urd bean (*V. mungo* (L) Hepperi) and their putative progenitors (*V. radiata* var. *sublobata* (Roxb.). Verdcourt and *V. mungo* (L.) var. *silvestris* Lukoki, Marechal and Otoul, respectively. In *V. radiata* var. *sublobata*, the number of bands varied from 25-29, while *V. radiata* showed the presence of 27 bands. The position of bands and Rf values were also corresponding very well. Similarly, in wild forms of *V. mungo* var. *silvestris*, the number of protein bands were 26-27 which differed very little from cultivated *V. mungo* with the exception of only few additional bands. Other wild forms of *V. umbellata, V. aconitifolia* and *V. trilobata* originating from diverse ecological regions/habitats showed the presence of relatively fewer number of bands.

Cultivated rice bean (*V. umbellata*) possessed 16 and 25 bands while wild forms exhibited activity with 18 bands only.

It was also evident from the results that intraspecies variation was very little, while intraspecies similarity was quite high as was evident from the similarity index (S.I.) value of 70.9 in case of *V. mungo* var. *silvestris* (PlX416 and PlX410). When compared with cultivated form (T_9) of urid bean (*V. mungo*), the wild type *V. mungo* var. *silvestris* (PlX410) resembled very much. It may be indicated here that PlX410 has been designated as the holotype specimen of *V. mungo* (L.) var. *silvestris*. (Lukoki, Marechal and Otoul, 1980). This phylogenetic relationship was also clearly established earlier by Chandel (1980) and Chandel *et al.* (1984). Similarly, several accessions of wild *V. radiata* var. *sublobata* (Roxb.) Verdcourt, exhibited close resemblance with mung bean cultivars **Pallavi** supported by similarity index (S.I. value 48.5-55.5%). Among other species, *V. trilobata* possessed some affinity with mung bean and its wild putative progenitor forms.

The data presented in Table 4 indicates the number and relative mobility of two enzymes peroxidase and catalase. In case of peroxidase, the Rf values among the various genotypes showed that slowest migrating band had a value of 0.18, while the fastest migrating band had 0.57. In case of catalase isoenzyme the values of slowest and fastest migrating bands were 0.16 and 0.30, respectively. It was unexpected to find that wild occurring putative forms of *V. umbellata, V. vexillata, V. dalzaliana* and *V. aconitifolia* exhibited no enzymatic activity at all for the catalase enzyme. The migration rates of different bands of the two enzymes (Table 4; Fig. 2) show that no intraspecies variations appeared. *V. mungo* var. *silvestris,* however, exhibited some similarity with *V. umbellata* for peroxidase enzyme and wild form of *V. aconitifolia* collected from Hyderabad region for peroxidase isoenzyme activity. Interestingly, *V. mungo* var. *silvestris* differed from rice bean (*V. umbellata*) for the catalase enzyme as the latter species did not exhibit any enzymatic activity. All the accessions of *V. trilobata* showed only the presence of one catalase band. While different forms/accessions of *V. trilobata* possessed three enzyme bands of catalase. *V. dalzaliana* resembled *V. trilobata* in having three enzyme bands and the position of each band also corresponded very well. The two bands of peroxidase in case of *V. vexillata* were entirely different from *V. trilobata, V. mungo* var. *silvestris, V. radiata* var. *sublobata* as well as *V. umbellata* and *V. aconitifolia.*

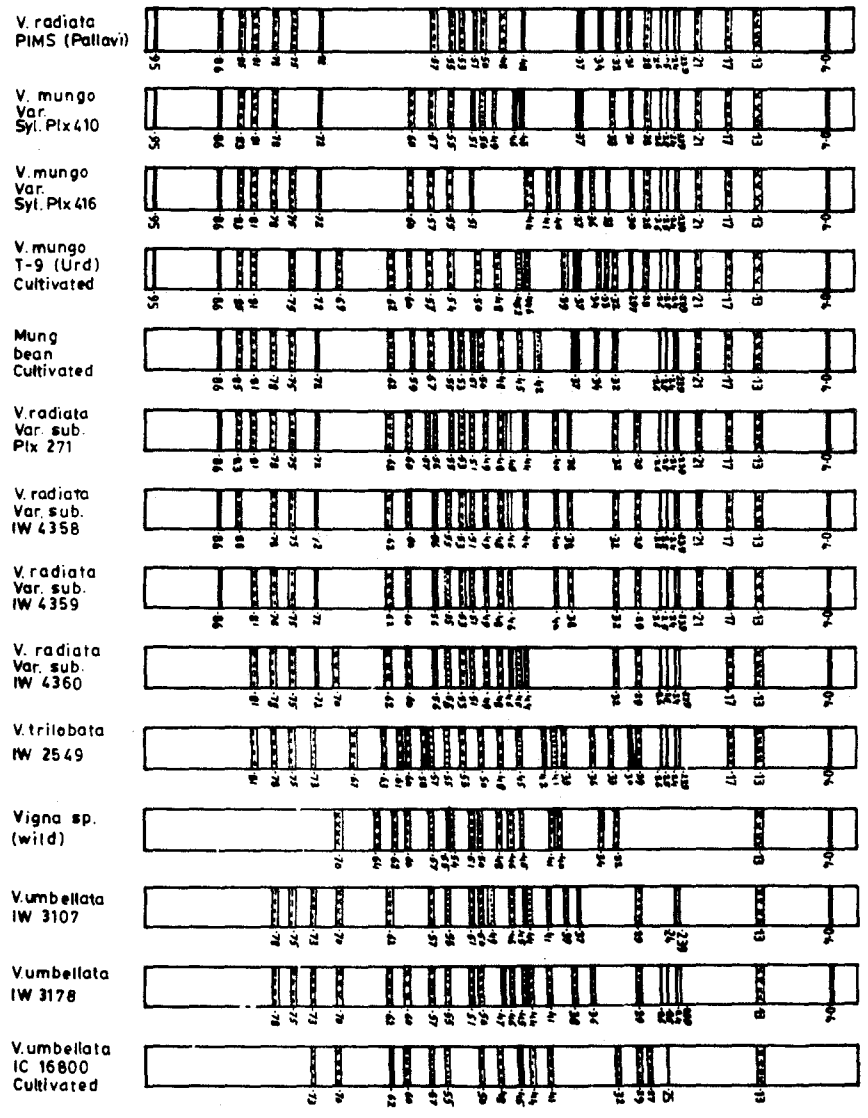

Fig. 2. Isozymes profiles of different accessions in Vigna species et al.

Table 4 : Number of peroxidase and catalase Bands and their R_f values determined for different genotypes of *Vigna* species by Slab Gel Electrophoresis using leaves

Genotype	Peroxidase				Catalase		
	No. of bands	Rf values			No. of bands	Rf values	
V. mungo var. *silvestris*							
" – IW 3385	1	0.57	—	—	1	0.30	—
" – IW 3386	1	0.57	—	—	1	0.30	—
" – IW 3387	1	0.57	—	—	1	0.30	—
" – IW 3388	1	0.57	—	—	1	0.30	—
" – IW 3389	1	0.57	—	—	1	0.30	—
" – IW 3393	1	0.57	—	—	1	0.30	—
" – Pl x 412	1	0.57	—	—	—	—	—
" – Pl x 413	1	0.57	—	—	1	0.30	—
" – Pl x 416	1	0.57	—	—	1	0.30	—
V. radiata var. *sublobata*							
" – Pl x 271	1	0.57	—	—	2	0.30	0.16
" – Pl x 272	1	0.57	—	—	2	0.30	0.16
" – Pl x 274	1	0.57	—	—	2	0.30	0.16
" – Pl x 278	1	0.57	—	—	2	0.30	0.16
" – Pl x 298	1	0.57	—	—	2	0.30	0.16
" – IW 4358	1	0.57	—	—	2	0.30	0.16
" – IW 4359	2	0.57	—	—	2	0.30	0.16
V. vexillata							
" – Pl x 401	2	0.51	0.21	—	—	—	—
" – IW 3426	2	0.51	0.21	—	—	—	—
" – IW 4347	2	0.51	0.21	—	—	—	—
V. dalzaliana							
" – Pl x 397	3	0.57	0.52	0.36	1	0.30	—
Vigna spp.							
" – Pl x 278	1	0.57	—	—	—	—	—
V. aconitifolia							
" – PLMO 211	—	—	—	—	1	0.30	—
" – PLMO 377	—	—	—	—	1	0.30	—
" – IC 9080	—	—	—	—	1	0.30	—
V. aconitifolia Wild putative (Type progenitor Hyderabad)	1	0.57	—	—	1	0.30	—
V. umbellata							
" – IW 3107	1	0.57	—	—	—	—	—
" – IW 3173	1	0.57	—	—	—	—	—
" – IW 3178	1	0.57	—	—	—	—	—
V. trilobata							
" – IW 4350	3	0.57	0.52	0.36	1	0.30	—
" – IW 4364	3	0.57	0.52	0.36	1	0.30	—
" – IW 4378	3	0.57	0.52	0.36	1	0.30	—
" – IW 2214	3	0.57	0.52	0.36	1	0.30	—
" – IW 2482	3	0.57	0.52	0.36	1	0.30	—
" – IW 4364	3	0.57	0.52	0.36	1	0.30	—
" – IC 24830	3	0.57	0.52	0.36	1	0.30	—
" – IC 24832	3	0.57	0.52	0.36	1	0.30	—
" – Pl x 92	3	0.57	0.52	0.36	1	0.30	—
" – Pl x 394	3	0.57	0.52	0.36	1	0.30	—
" – Pl x 511	3	0.57	0.52	0.36	1	0.30	—

Protein polymorphism in *Vigna* species was studied earlier by Chandel (1980) involving nine cultivated and wild species of *Vigna* of Asiatic origin. Using polyacrylamide gel electrophoresis the evolutionary relationships were conclusively elucidated amply supported by extensive study of comparative morphology, scanning electron microscopy of seed coat cellular and hilum morphology and leaf phenolic chromatography (Chandel *et al.*, 1984). The results analysed employing the numerical taxonomy techniques showed that evidences from all above independent studies, interestingly, arrived to the same conclusions. The results also convincingly identified the wild putative progenitors of mung and urid bean and showed that mung bean and urid bean are quite distinct species and are not closely related as contended by most earlier workers. In fact, these species have evolved independently, in India, from their widely occurring wild putative progenitors namely *V. radiata* (L.) var. *sublobata* (Roxb.) Verdcourt and *V. mungo* (L.) var. *silvestris* Lukoki, Marechal and Otoul, respectively. In the same year, parallel studies were carried out by Marechal's group in Belgium, who used one wild form (PlX410) in common and designated it as *V. mungo* var. *silvestris* Lukoki, Marechal and Otoul indicating similar relationships.

Using chromatography and seed protein electrophoresis in Ethiopean cereal *Eragrostis tef* (Bekele and Lester, 1981) and in *Festuca arundanacea* (Radomska and Lester, 1986) phylogenetic relationships were established. In latter case, morphological and seed protein studies of selected species of *Festuca* were carried out to elucidate the relationships between species of section *Ovineae* and their affinity with other fescue (Radomska and Lester, 1986).

Classical identification of cultivars and more so the germplasm diversity based on standard morphological markers has proved to be inadequate because of wide spectrum of phenotypic variation and their interaction with environment (Mannetji, 1984). In such instances, electrophoretic patterns illustrated through electrophorograms of seed proteins can be used effectively to decipher the similarities and differences between cultivars and genotypes (Bushuk and Zillman, 1978; Cooke 1984; and Hussain *et al.*, 1986).

Electrophoretic banding homology is considered to be a powerful approach in estimating genetic relationships between two or more species (Johnson, 1968; Johnson and Thein, 1970), as well as in the identification and classification of cultivars (Cooke and Draper, 1983). The techniques used in our studies have shown the

potential and usefulness in finding out the close affinities between various cultivated *Vigna* species and their several wild polymorphic forms. Electrophoresis together with other easily executable molecular methods on the proteins have the potential to supplement traditional methods of classification and thus provide strong basis for studying the finer relationships among organism at the species and subspecies level. The present study supports earlier view (Chandel, 1980; Chandel, 1984; Chandel *et al.*, 1984 and Chandel, 1991) with more detailed study on several accessions of *Vigna* and provides evidences in resolving prevalent confusion about species relationships in genus *Vigna*.

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