VEGETOS

Vol. 29 (Special) : 14-21 (2016)

ISSR Markers Analysis of Genetic Relationship Between Underutilized Beachpea [*Vigna marina* (Burm.) Merr.], Mungbean & Urdbean landraces of Bay Islands

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Published online: 05.02.2016
This article is published in open access at www.vegetosindia.org

Abstract

Bay islands or Andaman and Nicobar Islands are a unique insular habitat in Bay of Bengal with warm and humid climatologically features and ideal tropical ecological conditions. These islands are immensely rich in plant biodiversity and it is also hub of diversity for so many cultivated and wild species of pulses like greengram, blackgram, mungan, beachpea, ranmug, redgram, cowpea etc. Understanding the genetic relativeness between related species is one of the important criteria for effective utilization of germplasm in the crop breeding programme. This can be revealed by the analysis of intraspecific genetic relativeness in individual accessions of related taxa. The underutilized legume *Vigna marina* and promising landraces of mungbean and urdbean collected from the different parts of the Andaman and Nicobar Islands were characterized to obtain genetic relationships between these taxonomic entities using ISSR markers. Seven mungbean landraces and cultivars, 4 urdbean landraces and 4 accessions of beachpea [*Vigna marina* (Burm.) Merr] were analysed using 24 randomly selected ISSR markers. Seven ISSR markers were produced clear amplification profiles. The analysis was more discriminating and 65 polymorphic bands out of total 265 bands and 66 polymorphic bands out of total 335 bands were generated with seven ISSR markers. Based on the presence or absence of bands, Jacquard's similarity index was calculated to construct a dendrogram to show genetic distance between and within the accessions. Similarity index values ranged from 0.92 to 0.67. Matrices derived from ISSR data were used to construct UPGMA dendrograms. The dendrograms derived from ISSR data showed two main groups and four sub groups. In the tree obtained from accessions one *Vigna marina* were mixed with mungbean and urdbean accessions while, three clustered separately. The results indicate that ISSR markers can be effectively used in determination of genetic relationship among mungbean, urdbean and beachpea. It can be concluded that, the information of genetic relationship among underutilized pulse crops beachpea is closely related with mungbean and urdbean. Hence, it can be utilized for the development of desired crop varieties for the development new salt tolerant varieties of cultivated mungbean and urdbean crops.

Keywords: *Vigna marina,* mungbean, urdbean, ISSR markers, genetic relationship

Introduction

Pulses represent one of the most important foods that have been extensively used to cover basic protein and energy needs in day today life. Pulses have unique property of maintaining and restoring soil fertility through biological nitrogen fixation as well as covering and improving physical properties of soil by virtue of their deep root system. The genus *Vigna* is a pan-tropical genus comprising 104 legume species distributed widely in tropical and subtropical regions (Lewis *et al*. 2005) includes several species of economic importance. *Vigna marina* (Burm.) Merr., is a wild relative of cultivated *Vigna* species (Sanjeewani *et al*., 2012) distributed throughout the tropics and shows a great similarity in floral structure of mungbean and urdbean as the main distinctive characters being some vegetative traits possess chromosome number of 2n=22 (Verdcourt, 1971, Marechal *et al*., 1978). In particular, beachpea possess succulent stems and leaflets, the later being ovate, its legume-grain are broader, more glabrous and the seeds are oval

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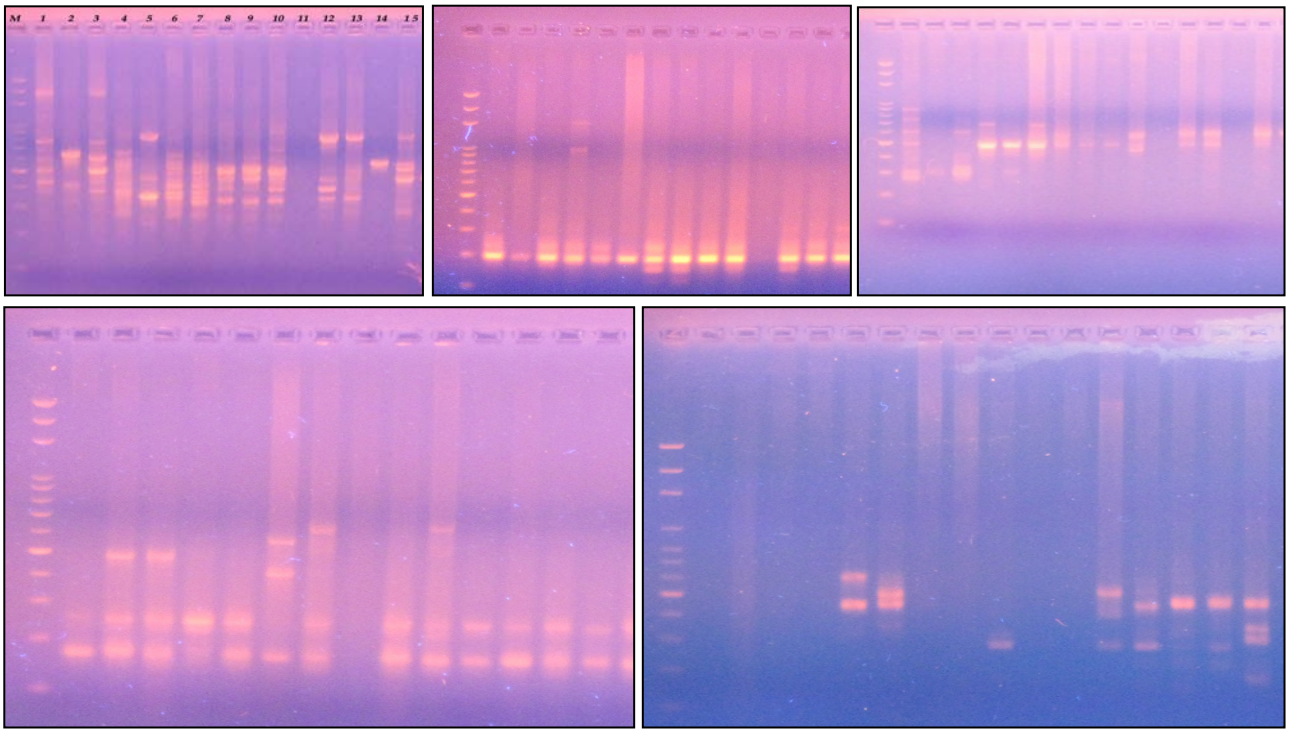
Fig 1. ISSR fingerprints of the mungbean, urdbean and beachpea (*Vigna marina*)

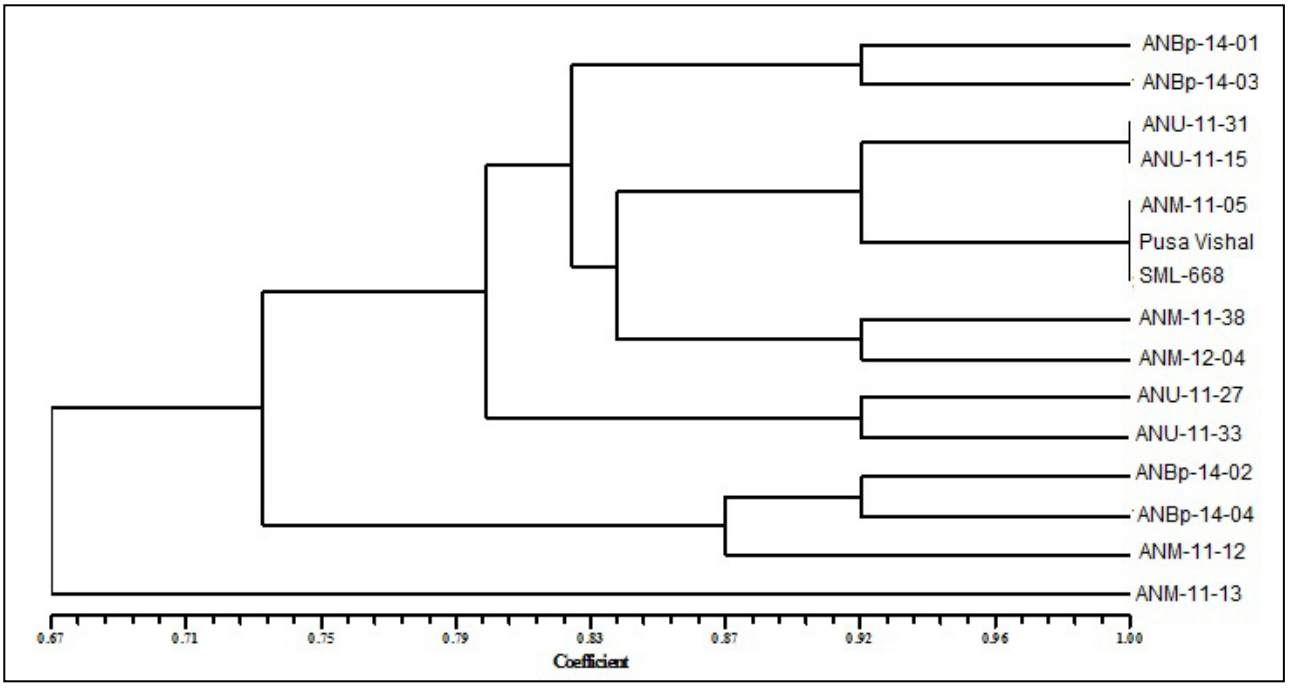


in shape, brown and radish brown in colour and larger than those of cultivated mungbean and urdbean. These all three legumes, namely mungbean, urdbean and beachpea differ in their habitats, as mungbean and urdbean grows along fresh water and are cultivated for their economic use.

The habitat of *Vigna marina* are associated with seashore where it may occur from immediately above watermark to low coastal dunes and scrub, particularly over sandstone and sandy soils. This is underutilized potential crops having protein content varies from 19.8 – 30.26 per cent with good source iron, calcium, magnesium and zinc in the fresh and dry seeds. This crop grows well sandy beaches in tropical and sub tropical regions (Chankaew, 2014 and Singh *et al.* 2014). It is generally found on tropical beaches around the world, such as those in Hawaii and on various islands in the Pacific Ocean, and other Caribbean Islands, the coast of Bahia, Brazil, the Atlantic and Indian coasts of Africa, Madagascar, the Seychelles, India (Andaman & Nicobar Islands) and Sri Lanka, Indo-china and the Chinese Island of Hainan, Malaysia, and along the Australian coast in Queensland and the Northern Territory (Verdcourt 1971, Marechal *et al.* 1978, Padulosi and Ng 1990, Padulosi and Ng 1993, Abraham *et al.* 2008, Elanchezhian *et al.* 2009, Singh *et al.* 2015). *Vigna marina* is determinate type, dune creeper having salt tolerant capacity (Elanchezian *et al.* 2009 and Sanjeewani *et al.* 2012). *Vigna marina is* prostrate, creeping vine, perennial underutilized pulse crop which is also known as the nanea, wild mung, beachpea and notched cowpea belongs to the family Fabeacae. The nitrogen-fixing ability of this crop provides a ready source of free fertilizer in the form of nitrogen to other plants growing in the area. This vine is great for open, sunny areas as a ground cover and especially good for beach front properties.

The Andaman-Nicobar group of islands is considered to be a veritable storehouse of plant biodiversity. Situated between two major biodiversity hotspot, namely the Indian subcontinent and the Malaysian-Indonesian region, it is hardly surprising that the Islands manifest biodiversity of extraordinary range within a limited geographical area. The flora of the Andaman group of islands shows closer affinity to the Indo-Myanmarese-Thai flora, while the Nicobar groups of islands are closer to the flora of Malaysia-Indonesia (Balakrishnan and Ellis 1996). Among pulse crops, much diversity occurs in wild forms of green gram, black gram mungan, beachpea, ranmug, redgram, cowpea etc. and the diversity of cultivated pulse crops is presently maintained in home gardens by settlers from the mainland and other adjoining countries (Abraham *et al.* 2008, Mandal and Majumdar 2014, Singh *et al.* 2014, Singh *et al.* 2014). The local holding of mungbean, urdbean landraces germplasm at the ICAR-Central Island Agricul-

Fig 2. UPGMA dendogram indicating the genetic relationship among mungbean, urdbean and beachpea (*Vigna marina*)



tural Research Institute, Port Blair also includes wild relative's accessions beachpea (*Vigna marina*) for their agro-morphological characterization and utilization in pulse improvement programme has not been clearly determined. While, the analysis of genetic relationship with cultivated species is equally important for further enhancement of collection. Since, a very few studies on the analysis of the genetic relationship among *Vigna* cultivated species and their wild relatives has been done isozyme and RAPD markers (Sonnate *et al.* 1997, Samarjeewa *et al.*, 2002). Therefore, the present study was conducted to examine the genetic relationship between cultivated landraces of mungbean, urdbean and beachpea (*Vigna marina*) by using ISSR markers for further breeding programme.

Material and Methods

Plant material

The plant material used in this study includes randomly selected mungbean (07 accessions), urdbean (4 accessions) landraces collected from the farmers field of Andaman and Nicobar Islands and beachpea (4 accessions) (Table 1) collected from South Andaman and Car Nicobar. Five seeds of each accession were grown in pots and leaf samples were collected at the age of 15 days in case of cultivated landraces and 30 days age from the plants of beachpea (*Vigna marina*) for DNA isolation and analysis.

Plant DNA isolation

Fresh leaves from young plants were collected and frozen in liquid nitrogen. Leaves were ground with a mortar and pestle. DNA was isolated according to the cetyltrimethyl ammonium bromide (CTAB) protocol described by Murray and Thomson (1980), with slight modifications as described below. Up to 200 mg of ground leaf tissue was transferred to 2 ml eppendorf tubes, mixed with 500 µl of 2 × CTAB extraction buffer and incubated in a 65°C water bath with frequent agitation for 90 min. The tubes were removed from the water bath and allowed to cool until room temperature before 500 µl of phenol was added and mixed thoroughly. The mixture was centrifuged at 12,000 rpm for 10 min and the upper supernatant phase collected in a new tube. A second extraction was performed with 500 µl of a mixture of 24% of phenol/chloroform and 1% of isoamyl alcohol (v/v). After centrifugation, the supernatant was treated with RNase and the last extraction was performed with chloroform isoamyl alcohol. The upper phase was transferred into a new tube and DNA was precipitated with equal volumes of 2–propanol and sodium-acetate. The DNA pellet was washed with 70% ethanol and was dried for 5 min in a heating block of 60°C. The resulting DNA pellet was dissolved in 100 µl of distilled and sterilized water (SIGMA). DNA integrity was tested, using 1.5% agarose gel electrophoresis, and its concentration was determined with a UV spectrophotometer. DNA was then diluted to 25 ng/µl for PCR amplification.

Table 1. A brief description of different mungbean, urdbean landraces and beachpea genotypes

Accession Code	Crop/ wild relatives/ species	Genotypic entity	Place of Collection
ANBp-14-01	Beachpea/ *Vigna marina*	Landrace	Chidiyatapu, South Andaman
ANBp-14-02	Beachpea/ *Vigna marina*	Beachpea landrace	Manjery, South Andaman
ANBp-14-03	Beachpea/ *Vigna marina*	Beachpea landrace	Car Nicobar, Nicobar
ANBp-14-04	Beachpea/ *Vigna marina*	Beachpea landrace	Car Nicobar, Nicobar
ANM-11-05	Mungbean/ *Vigna radiata*	Mungbean Landrace	Khudirampur, North & Middle Andaman
ANM-11-12	Mungbean/ *Vigna radiata*	Mungbean Landrace	Nabagram, North & Middle Andaman
ANM-11-13	Mungbean/ *Vigna radiata*	Mungbean Landrace	Nabagram, North & Middle Andaman
ANM-11-38	Mungbean/ *Vigna radiata*	Mungbean Landrace	Khudirampur, North & Middle Andaman
ANM-12-04	Mungbean/ *Vigna radiata*	Mungbean Landrace	Bhartiya Nagar, Little Andaman
Pusa Vishal	Beachpea/ *Vigna marina*	Improved variety	Division of FCIP, CIARI, Port Blair
SML 668	Mungbean/ *Vigna radiata*	Improved variety	Division of FCIP, CIARI, Port Blair
ANU-11-15	Urdbean/ *Vigna mungo*	Urdbean landrace	Nayagarh, North & Middle Andaman
ANU-11-27	Urdbean/ *Vigna mungo*	Urdbean landrace	Madhupur, North & Middle Andaman
ANU-11-31	Urdbean/ *Vigna mungo*	Urdbean landrace	R.K. Gram, North & Middle Andaman
ANU-11-33	Urdbean/ *Vigna mungo*	Urdbean landrace	Bhartiya Nagar, Little Andaman

ISSR analysis

Selection of ISSR primers

In order to select primers, that can give polymorphic DNA products, ISSR analysis was carried out using 24 randomly selected ISSR primers. Seven out of thirty primers gives amplified products were selected for evaluation of genetic relationship between mungbean, urdbean and *Vigna marina*.

DNA amplification

ISSR amplification reactions were carried out in 15 ml volume containing 25 ng template DNA, 0.5 units of Taq DNA polymerase, 0.1 mM dNTP each, 10mM primer, 1X reaction buffer and distilled de- ionized water. The PCR amplification was done using the Thermalcycler (Biorad, USA) with an initial denaturation step of 5 min at 94 °C, followed by 45 cycles at 940C for 1 min, 48 0C- 53 0C (depending upon the primer pair) for 2 min, 72 0C for 1 min and final extension 1 cycle of 720C for 10 minutes. PCR amplified products were subjected to electrophoresis in a 3% agarose gel in 1x TBE buffer at 80 v for 3 hours. Ethidium bromide stained gels were documented using Alpha Imager TM 1200.

Statistical analysis

The binary data of marker genotype matrix was used for analysis using NTSYS pc (Numerical taxonomy system, version 2.02, Rohlf, 2000). The SIMQUAL programme was used to calculate the Dice coefficient. The marker data was then standardized for principal coordinate analysis (PCoA) using NTSYS pc software to highlight the resolving power of the ordination of Shannon index. The efficiency of primers to bring out the genetic diversity was estimated by Shannon index. The Shannon index was calculated as $H = - \Sigma Pi \ln Pi$, in which pi is the frequency of a given ISSR fragment.

Results

Twenty four selected ISSR primers were used to analyze the 15 mungbean, urdbean and beachpea genotypes. The PCR amplification using ten, 5'-anchored dinucleotide repeat primers gave rise to reproducible amplification products. ISSR primers produced varying number of DNA fragments, depending on their ISSR patterns. All 7 scorable ISSR primers studied were polymorphic and a total of 335 scorable markers were generated, of which 66 were polymorphic among the 15 genotypes. Among the ISSR primers, UNC-827 was found to amplify the highest number of ISSR fragments followed by UBC-851, ISSR-24, ISSR-22 and ISSR-18, while produced the least number of ISSR fragments were produced by UBC-840. ISSR markers profiles produced by the 7 ISSR primers shown in Fig 1 produced 335 clear and distinct bands across 15 genotypes, of which 66 were polymorphic with an average of 47.85 / primer with 0.499 PIC value. The total number of amplified bands produced by each primer varied from 20 by primer UBC840 to a maximum of 106 by UBC -827. The maximum number of polymorphic bands was also produced by ISSR primer UBC -827 (18 number of poly-

Table 2. Code and sequence of the seven ISSR primers used for identifying the amplified DNA bands in mungbean, urdbean and beachpea (*Vigna marina*)

Primer	Sequence	Total number of bands	Total number of polymorphic bands	% of polymorphism	PIC
ISSR22	(CA)6AC	45	8	17.77	0.468
UBC840	(GA)8YT	20	6	30.00	0.485
ISSR24	(GA)6CC	46	11	23.91	0.250
UBC851	(GT)8YT	49	7	14.28	0.330
ISSR18	(AAC)5	43	9	20.93	0.375
ISSR14	(GACA)4	26	7	26.92	0.485
UBC827	(AC)8G	106	18	16.98	0.250
Total		**335**	**66**		

morphic bands) while, minimum polymorphic bands were produced by UBC 840 (6 number). The size of amplified bands also varied with different primers and it ranged from 3000 to 200 bp. Similarity coefficients values for the 7 mungbean, 4 urdbean and 4 beachpea genotypes based on ISSR markers ranged from 1.00 between urdbean landraces (between ANU-11-31 and ANU-11-15), mungbean landraces (between ANM-11-05, Pusa Vishal and SML-668) to 0.67 between ANM-11-13 and other genotypes of mungbean, urdbean and beachpea.

The ISSR data were used to generate UPGMA dendogram (Fig 2). Cluster analysis revealed that the genotypes are grouped into four clusters with 67% similarity. The present study has shown that the genotypes of beachpea (*Vigna marina*) ANBp-14-02 and ANBp-14-04 collected from Manjery of South Andaman and Car Nicobar showing 87 % similarity with mungbean landrace ANM-11-12 collected from North Andaman. Cluster analysis revealed that the genotypes are grouped into three clusters with 53% similarity and it has shown that the genotypes of *Vigna marina* collected from various islands showing 90 % similarity among and with mungbean and urdbean. The results indicated that ISSR individually can be effectively used in determination of genetic relationship among *Vigna* species. The highest amount of polymorphism was exhibited by ISSR-14 and ISSR-24. Alleles produced by different primers ranged from 7to 11 with an average of 8.75 per primer and the level of polymorphism was found to be 22.38 percent. The Shannon indices varied from 0.250 (ISSR-24) to 0.485 (ISSR-14) with an average value of 0.39 indicating high resolving power of the ISSR markers. In the present study, ISSR markers detected 22.38% polymorphism. ISSR markers have been successfully utilized for analysis of pattern of genetic relationships in greengram. The results indicated that ISSR markers have been successfully utilized for assessing genetic relationship and revealed remarkable molecular discrimination between the 7 mungbean, 4 urdbean and 4 beachpea genotypes. Similar results were reported by Hady *et al.*, 2010 where they reported that RAPD and ISSR markers individually or in combination can be effectively used in determination of genetic relationship among *Vigna* species and Ajibade *et al.*, 2000, where they found that the ability of ISSR technique to be effectively distinguish species in the genus *Vigna*.

Discussion

The mungbean, urdbean landraces and wild relative beachpea (*Vigna marina*) constitutes a rich source of agro-biodiversity of these islands and their conservation and utilization requires that their genetic structure is well characterized and understood. Molecular characterization using DNA is a routine method employed to study the extent of genetic relativeness across the set of germplasm or cultivars and group them into specific categories. Comparative studies in *Vigna* species involving RAPD, AFLP, ISSR and SSR marker systems were successfully used by very limited researchers (Souframanien and Gopalakrisna 2004, Gillaspie *et al.* 2005). With a number of studies it has been shown that molecular characterization can reveal the maximum genetic relatedness found in population. The present study also shows that ISSR markers can be useful to supplement the morphological traits in the analysis of genetic relationship not only between species but also between the accessions of the same species. Although, Kaga *et al.*, 2005 and Hady *et al.*, 2010 have been employed molecular markers for analyzing samples from *Vigna* species. In the present study genetic relationship of mungbean (*Vigna ra-*

Table 3. Similarity coefficients of the mungbean, urdbean and beachpea landraces based on ISSR markers

Case	Genotypic Matrix														
	ANBp-14-01	ANBp-14-02	ANBp-14-03	ANBp-14-04	ANU-11-27	ANU-11-33	ANU-11-31	ANU-11-15	ANM-11-13	ANM-11-12	ANM-11-38	ANM-12-04	ANM-11-05	Pusa Vishal	SML668
ANBp-14-01	1.000														
ANBp-14-02	0.583	1.000													
ANBp-14-03	0.916	0.666	1.000												
ANBp-14-04	0.666	0.916	0.583	1.000											
ANU-11-27	0.750	0.666	0.833	0.583	1.000										
ANU-11-33	0.833	0.583	0.750	0.666	0.916	1.000									
ANU-11-31	0.833	0.750	0.916	0.666	0.916	0.833	1.000								
ANU-11-15	0.833	0.750	0.916	0.666	0.916	0.833	1.000	1.000							
ANM-11-13	0.833	0.583	0.750	0.666	0.583	0.666	0.666	0.666	1.000						
ANM-11-12	0.750	0.833	0.666	0.916	0.666	0.750	0.750	0.750	0.750	1.000					
ANM-11-38	0.833	0.750	0.750	0.833	0.750	0.833	0.833	0.833	0.666	0.916	1.000				
ANM-12-04	0.916	0.666	0.833	0.750	0.666	0.750	0.750	0.750	0.750	0.833	0.916	1.000			
ANM-11-05	0.750	0.833	0.833	0.750	0.833	0.750	0.916	0.916	0.583	0.833	0.916	0.833	1.000		
Pusa Vishal	0.750	0.833	0.833	0.750	0.833	0.750	0.916	0.916	0.583	0.833	0.916	0.833	1.000	1.000	
SML668	0.750	0.833	0.833	0.750	0.833	0.750	0.916	0.916	0.583	0.833	0.916	0.833	1.000	1.000	1.000

diata), urdbean (*Vigna mungo*) with *Vigna marina* agree with those records. Grouping patterns generated by cluster analysis based on molecular analysis showed the genetic diversity between genetically related mungbean, urdbean and beachpea (Singh *et al.* 2014, 2015). The present study reveals that, the inclusion of genotypes bred for specific objectives like salinity tolerance (Elanchezian *et al.* 2009 and Sanjeewani *et al.* 2012) resulting in narrowing of genetic base and the marker system used could be the reason for clustering most of the cultivars in one cluster. The relationships between the landraces are not necessarily reflecting the agronomic traits. Molecular markers are scattered throughout the genome and their association with various agronomic traits is influenced by the cultivator under selection pressure induced by domestication. It could be concluded that, the information of genetic similarities among cultivated landraces of mungbean, urdbean and cowpea with *Vigna marina* and this wild relatives can be utilize as the potential source of genetic resources further breeding programs in developing salt tolerant desirable genotypes of *Vigna* species particularly in mungbean and urdbean. Exploration and evaluation of diversity among these landraces would be of great significance for *in situ* conservation and rice bean breeding programmes.

Acknowledgements

Authors are grateful to Indian Council of Agricultural Research, Ministry of Agriculture & Farmers Welfare, Government of India, New Delhi for providing financial support. We are also grateful to Dr. S. Dam Roy, Director, ICAR - Central Island Agricultural Research Institute, Port Blair (Andaman & Nicobar Islands) for all kinds of support and encouragement towards research and development activities carried out.

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