Ricebean Network

European Commission 6th Framework Programme, Project no. 032055

FOSRIN

Food Security through Ricebean Research in India and Nepal

Report 7: Molecular diversity in ricebean

MOLECULAR MARKER DIVERSITY OF NEPALESE AND INDIAN RICEBEAN GERMPLASM

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EXECUTIVE SUMMARY

Ricebean [*Vigna umbellata* (Thunb) Ohwi and Ohashi] is a grain legume, locally important in parts of India and in Nepal for the food security and nutritional well-being of poor farmers in marginal areas. It is an under-utilised crop, cultivated in marginal hill areas of India and Nepal, often as an intercrop with maize and other grain legumes.

We studied genetic diversity in molecular marker traits of 112 ricebean genotypes using selective simple sequence repeats (SSR) of adzuki bean (*V. angularis*). The germplasm comprised 91 landrace accessions from Nepal and 21 from India, and represented a wide geographical distribution in both countries. Our objective in obtaining information on this genetic diversity was to subsequently relate it to the morphological and biophysical diversity of ricebean.

Thirty-five SSR primer pairs (out of 109 Adzuki bean SSRs earlier screened and identified as polymorphic loci) were used to characterise genotypes. Variations in alleles as traits were recorded and generated the molecular data. The allelic molecular data were analysed to determine the diversity indices: number of alleles per locus, number of alleles per polymorphic locus, percentage of amplified primers, percentage of polymorphic markers (PPL), percentage of polymorphic alleles (PPA) and polymorphic information content (PIC) of each primer. The relationships between the Nepalese and Indian accessions were subjected to principal component (PCA) and cluster analyses.

Among 35 primer pairs analysed in the Nepalese accessions, 30 loci (86%) revealed polymorphism with polymorphic information content (PIC) values ranging from 0.03 to 0.63 with an average of 0.25. In the Indian germplasm 30 loci (73%) revealed polymorphism, with PIC from 0.08 to 0.70 (average 0.27). Estimates of diversity as Shannon Weaver indices (SW) were similar for both the Nepalese and the Indian accessions at 0.47 and 0.48 respectively. A total of 80 alleles (bands) were identified for 30 loci across the 112 accessions, in which 94% and 85% of polymorphic alleles were detected with an average of 2.5 and 2.1 alleles per polymorphic locus respectively in the Nepalese and the Indian accessions. Indian accessions were closely related and grouped together in the PCA scatter plot, while the Nepalese genotypes were dispersed and showed comparatively greater genetic variation. However, the magnitude of genetic differentiation measured as diversity index (H') and SW values were similar (range 0.25-0.27 and 0.47-0.48 respectively). The genetic similarity among all accessions, including the checks, was 0.5835, and their relationships were shown in a dendrogram. The Indian genotypes were tightly clustered together and occurred in between the bold-seeded ricebean and the small-seeded adzuki bean types of Nepalese accessions.

We show a remarkable level of diversity in the microsatellite markers. Genotypes were characterised for molecular marker traits, and the work suggested that adzuki bean SSR markers could be used in germplasm analysis and for assessing genetic diversity in ricebean. The polymorphic SSR loci with AG repeats were most informative, while the information on genetic variation could be useful in conservation and crop improvement. The study also provided evidence that microsatellite markers could be developed using ricebean genomic DNA.

Series Editor: PA Hollington

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Correct citation: Bajracharya, J; Singh, S; Dangol, B; Hollington, PA & Witcombe, JR (2010) Food Security through Ricebean Research in India and Nepal (FOSRIN). Report 7. Molecular marker diversity in Nepalese and Indian ricebean germplasm (Ed PA Hollington). Kathmandu, Nepal, Nepal Agricultural Research Council and Bangor, Wales, UK, Centre for Advanced Research in International Agricultural Development, College of Natural Sciences, Bangor University.

This document is an output from the project Food Security through Ricebean Research in India and Nepal (FOSRIN), funded by the European Commission under the 6th Framework Programme contract 032055. The opinions therein are those of the authors and may not be taken as representing those of the European Commission.

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Introduction

Biosystematics of ricebean and the genus Vigna

Ricebean [*Vigna umbellata* (Thunb) Ohwi and Ohashi] is an under-utilised grain legume cultivated in hill areas of India and Nepal. It is often under-cultivated as an intercrop with maize and other grain legumes. It is one of the eight *Vigna* species domesticated in Asia, and is very closely related to adzuki bean [*V. angularis* (Willd.) Ohwi and Ohashi] (Kaga *et al,* 1996; Tomooka *et al.,* 2003). The original centre of domestication of the Asian *Vigna* species, including ricebean, is thought to be Indo China, and hence it is a native species of South and Southeast Asia. It belongs to the subgenus *Ceratotropis,* which comprises 3 sections, *Ceratotropis*, *Aconitifoliae* and *Angulares* (Tomooka *et al.,* 1991). *Angulares,* to which ricebean belongs, is the most recent and diversified section of the subgenus (Doi *et al*., 2002). Ricebean is believed to be domesticated from the wild form *V. umbellata* var *gracilis,* which is cross fertile type (Lawn, 1979). These wild types have been reported to occur in natural and disturbed habitats and are indeterminate, photoperiod-sensitive, freely-branching, twining plants with small seeds. Ricebean landraces cultivated in Nepal are similar to these wild types (Tateshi, 1985). Ricebean is an annual vine legume with robust vegetative growth, and has a haploid chromosome number of 11 (2n = 2x = 22) (Chaitieng *et al.,* 2006).

Distribution and cultivation in Nepal and India

Ricebean is known for its diverse distribution and is adapted to a range of altitudes. It ranges from humid subtropical to warm and cool temperate regions of diverse agro-ecosystems, from the lowlands to the high hills of Nepal, and across the country from the east to the far west. In India, its distribution is mainly confined to the tribal regions of the North Eastern hills and to hill areas of the Western and Eastern Ghats (Arora *et al.,* 1980). Normally, ricebean is not grown on the main cultivable land of a holding, but on marginal and fallow land, slopes and bunds under rainfed conditions in maize-based cropping systems. However, in the lowlands it is also grown with rice in bunds. Ricebean in Nepal is most common between 700 and 1400 masl, but it is also found between 300 and 600 masl and even to 2400 masl in Humla, a high-hill district (Gautam *et al.,* 2007). In the North Eastern regions of India ricebean is important in the hill areas, and in the about 1.7 million ha under shifting cultivation, locally known as *Jhum*, where it could be a good option to increase productivity and improve the economics of farmers (Sarma *et al.,* 1995; Gupta *et al.,* 2009). Ricebean is photosensitive (Chaudhuri & Prasad, 1972; Chandel *et al*., 1988; Lokesh *et al.,* 2003, Sarma et al, 2003). It is normally a rainfed crop, sown in June-July (rainy season, summer season) with little or no inputs. It starts flowering during September and October when daylength begins to shorten, and is harvested in November or December.

Economic importance

Ricebean is an important food legume, particularly in the mid-hills of Nepal and Northern and Eastern hilly regions with shifting cultivation areas in India. It has a pivotal role as a pulse and as a supplementary food crop with potential economic importance in supporting the food security of the rural poor people in the regions. The crop also has important roles in animal and soil nutrition, as it is reported to be of high nutritional quality (16-25% protein, with an appreciable quantity of essential amino acids, vitamins and minerals) and restores soil fertility through biological nitrogen fixation (Lohani, 1979). The crop has excellent food and fodder values and is grown for fodder, green manure and cover crop. The dry seeds are eaten boiled as *dhal* (soup) and young immature pods are consumed as vegetables (Gupta *et al.,* 2009). Despite these values and advantages ricebean is little exploited, and most cultivation

and production is for local consumption. The crop is considerably neglected and productivity is very low, averaging around 200-300 kg ha^{-1}.

Genetic diversity

The multiple needs of farming households, their socioeconomic resources, and the prevalent agro-ecological conditions have greatly supported the conservation of crop diversity in the form of landraces in both Nepal and India. Ricebean is an under-utilised and unexploited crop and also an important cultigen. Diverse landraces with different plant and seed morphologies have been locally produced, maintained, and built up by farming communities over the generations.

Variability in legumes is largely based on their morphology, agronomic behaviour, and biochemical traits. There is a limitation on this traditional characterisation, as it is associated with a low level of polymorphism in legumes (Yamaguchi, 1992; Lumpkin & McClary, 1994). Asian *Vigna* have largely been studied for their molecular marker diversity including the traditional agro-morphological diversity. Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNAs (RAPDs), inter simple sequence repeats (ISSRs), and simple sequence repeats (SSRs) are molecular marker techniques that have been extensively used in genome analysis in Asian *Vigna*, especially adzuki bean. Construction of linkage maps and phylogenetic relationships and genetic diversity studies have been carried out in cultivated and natural species of *Vigna* including subgenera *Ceratotropis* (Kaga, 1996; Tomooka *et al.,* 1998; Yee *et al.,* 1999; Ajibade *et al.,* 2000; Xu *et al.,* 2000; Souframanien & Gopalkrishna, 2004; Han *et al.,* 2005; Seehalak *et al.,* 2006). Linkage maps have been developed for three of the Asian *Vigna* species: mung bean (*V. radiata*), adzuki bean, and black gram (*V. mungo*) (Kaga *et al.,* 2005; Chaitieng *et al.,* 2006) and a large number of SSR markers have been developed for adzuki bean (Wang *et al.,* 2004; Han *et al.,* 2005). These SSR markers have been used to develop comparative linkage maps with other related legumes and have provided information on genetic relationships among related species. SSRs or microsatellites are DNA sequences with repeat lengths of a few base pairs. Using them as molecular markers combines many desirable properties, and they have become the preferred technique for a wide range of applications in genetic mapping and genome analysis, genotype identification, variety protection, seed purity evaluation, and germplasm conservation (Brown *et al.,* 1996).

Objective

Our objectives were:

- to characterise Indian and Nepalese ricebean germplasm for SSR molecular marker traits,
- to investigate genetic variation and analyse the genetic diversity of ricebean germplasm using SSR molecular marker data,

Materials and methods

Plant materials

Studies to determine the molecular marker diversity in Nepalese ricebean germplasm were carried in the Biotechnology Unit, Nepal Agriculture Research Council (NARC), Khumaltar, Nepal and for Indian germplasm in the Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, India, during 2008 and 2009. Ninety one accessions of ricebean from Nepal and 21 from India including three checks: a bold (large) grained genotype named '*Masyang'* collected from the east of Nepal, a small-seeded genotype named '*Gurans'* collected from the far west of Nepal, and an accession of adzuki bean were used.

These accessions represented the stratified random samples of ricebean from Nepal and India. They were collected from different districts of Nepal between 1976 (conserved in the national genebank by NARC at Khumaltar, Nepal) and 2006 (Figure 1). These accessions were selected from 218 NPGR and LRGR germplasm accessions, based on passport and agro-morphological diversity carried out in different production environments in 2006 and 2007 in Nepal. These genotypes were diverse in local names, collected from districts representing the agro-ecological range, growth habit and seed characteristics. The accession number, the sites representing the collection districts and the agro-ecological range and year of collection of these stratified samples are presented in Table 1. Similarly, the Indian accessions were collected from different States and districts within them, and represent the stratified random samples selected based upon agro-morphological diversity.

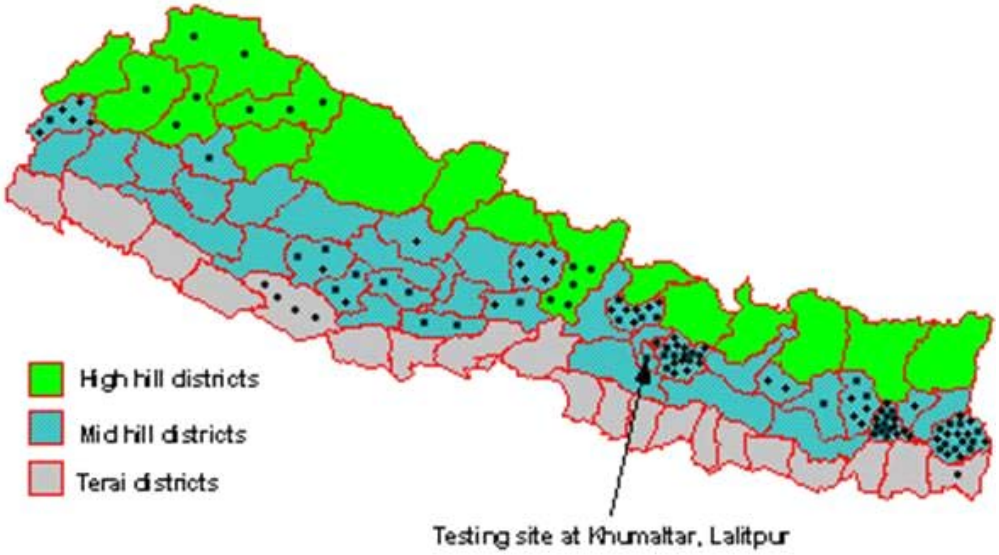


Figure 1: Ricebean germplasm and its collection from different agro-ecosystems representative of districts of Nepal

Table 1: List of stratified random samples of ricebean used in SSR marker diversity

Accession number	Collection districts	Ecological classification	Local name	Remarks
NPGR-00007	Nuwakot	Mid-hill, Central Nepal	*Chhirbire masyang*	Core collection evaluation
NPGR-00008	Nuwakot	Mid-hill, Central Nepal	*Panhelo masyang*	Included in mother trial in 2008
NPGR-00010	Lalitpur	Mid-hill, Central Nepal	*Masyang*	Core collection evaluation
NPGR-00012	Nuwakot	Mid-hill, Central Nepal	*Rato masyang*	Core collection evaluation
NPGR-00015	Bhaktapur	Mid-hill, Central Nepal	*Masyang*	Included in mother trial in 2008
NPGR-00073	Gulmi	Mid-hill, Western Nepal	*Thulo panhelo masyang*	Core collection evaluation
NPGR-00076	Arghakhanchi	High-hill, Western Nepal	*Dhawanse masyang*	Included in mother trial in 2008
NPGR-00087	Pyuthan	High-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-00090	Dang,	Terai, Western Nepal	*Jhilinge masyang*	Core collection evaluation
NPGR-00194	Kabre	Mid-hill, Central Nepal	*Masyang*	Included in mother trial in 2008
NPGR-01975	Baitadi	Mid-hill, Western Nepal	*Baramase masyang*	Core collection evaluation
NPGR-05364	Bhojpur	High-hill, Eastern Nepal	*Masyang*	Included in mother trial in 2008
NPGR-05368	Bhojpur	High-hill, Eastern Nepal	*Bhage masyang*	Core collection evaluation
NPGR-05370	Terhathum	High-hill, Eastern Nepal	*Rato ghore*	Core collection evaluation
NPGR-05373	Gorkha	High-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-05377	Lamjung	Mid-hill, Western Nepal	*Gurans*	Core collection evaluation
NPGR-05382	Tanahu	Mid-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-05384	Mugu	High-hill, Western Nepal	*Gurans*	Core collection evaluation
NPGR-05386	Humla	High-hill, Western Nepal	*Gurans*	Core collection evaluation
NPGR-05391	Bajura	Mid-hill, Western Nepal	*Ghore mas*	Core collection evaluation
NPGR-05396	Illam	Mid-hill, Eastern Nepal	*Banmara masyang*	Core collection evaluation
NPGR-05420	Dhankuta,	Mid-hill, Eastern Nepal	*Ghore mas*	Included in mother trial in 2008
NPGR-05423	Dhankuta	Mid-hill, Eastern Nepal	*Seto mas*	Core collection evaluation
NPGR-05432	Baitadi	Mid-hill, Western Nepal	*Gurans*	Core collection evaluation
NPGR-05565	Okhaldhunga	High-hill, Eastern Nepal	*Masyang*	Core collection evaluation
NPGR-06591	Mugu	High-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-06657	Kalikot	High-hill, Western Nepal	*Rato masysng*	Core collection evaluation

Accession number	Collection districts	Ecological classification	Local name	Remarks
NPGR-06756	Humla	High-hill, Western Nepal	*Gurans*	Included in mother trial in 2008
NPGR-07583	Jhapa	Tarai, Eastern Nepal	*Masyang*	Core collection evaluation
NPGR-07882	Bajhang	High-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-08380	Myagdi	Mid-hill, Western Nepal	*Syaltung*	Core collection evaluation
NPGR-08382	Banglung	Mid-hill, Western Nepal	*Syaltung*	Core collection evaluation
NPGR-09391	Syangja	Mid-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-09461	Panchthar	High-hill, Eastern Nepal	*Masyang*	Core collection evaluation
NPGR-09464	Taplejung	High-hill, Eastern Nepal	*Masyang*	Core collection evaluation
LRGR-42	Surkhet	Mid-hill, Western Nepal	*Siltung*	Core collection evaluation
LRGR-43	Surkhet	Mid-hill, Western Nepal	*Siltung*	Core collection evaluation
LRGR-44	Surkhet	Mid-hill, Western Nepal	*Siltung*	Core collection evaluation
LRGR-75	Pyuthan	Mid-hill, Western Nepal	*Raiyans*	Core collection evaluation
LRGR-91	Dang	Terai, Western Nepal	*Siltung*	Included in mother trial in 2008
LRGR-99	Palpa	High-hill, Western Nepal	*Jhilinge*	Included in mother trial in 2008
LRGR-101	Palpa	High-hill, Western Nepal	*Jhilinge*	Core collection evaluation
LRGR-102	Palpa	High-hill, Western Nepal	*Jhilinge*	Core collection evaluation
LRGR-103	Palpa	High-hill, Western Nepal	*Jhilinge*	Included in mother trial in 2008
LRGR-107	Palpa	High-hill, Western Nepal	*Jhilinge*	Core collection evaluation
LRGR-111	Gulmi	Mid-hill, Western Nepal	*Jhilinge*	Included in mother trial in 2008
LRGR-117	Gulmi	Mid-hill, Western Nepal	*Jhilinge*	Included in mother trial in 2008
LRGR-129	Palpa	High-hill, Western Nepal	*Siltung*	Core collection evaluation
LRGR-137	Kaski	Mid-hill, Western Nepal	*Masyang*	Core collection evaluation
LRGR-152	Kavre	Mid-hill, Western Nepal	*Masyang*	Core collection evaluation
NPGR-05435			*Gurans*	Non core collection evaluation
LRGR-7	Dadeldhura	Mid-hill, Western Nepal	*Gurans*	Non core collection evaluation
NPGR-06725	Humla	High-hill, Western Nepal	*Gurans*	Non core collection evaluation
LRGR-55	Baitadi	Mid-hill, Western Nepal	*Gurans*	Non core collection evaluation
LRGR-30	Bajura	Mid-hill, Western Nepal	*Gurans*	Non core collection evaluation
LRGR-8	Dadeldhura	High-hill, Western Nepal	*Gurans*	Non core collection evaluation
LRGR-3	Dadeldhura	High-hill, Western Nepal	*Gurans*	Non core collection evaluation
LRGR-79	Dang	Terai, Western Nepal	*Raiyans*	Non core collection evaluation
NPGR-00014	Nuwakot	Mid-hill, Central Nepal	*Dhade rato masyang*	Non core collection evaluation
FOSRIN-06	Kathmandu	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
NPGR-00183	Kavre	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
FOSRIN-02	Dailekh	Mid-hill, Western Nepal	*Siltung*	Non core collection evaluation
LRGR-90	Dang	Terai, Western Nepal	*Siltung*	Non core collection evaluation
LRGR-132	Gulmi	Mid-hill, Western Nepal	*Jhilinge*	Non core collection evaluation
NPGR-00091	Dang	Terai, Western Nepal	*Chhirbire masyang*	Non core collection evaluation
NPGR-00088	Pyuthan	High-hill, Western Nepal	*Chhirbire masyang*	Non core collection evaluation
NPGR-00188	Kavre	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
NPGR-05395			*Thulo rato ghore*	Non core collection evaluation
NPGR-05367	Bhojpur	High-hill, Eastern Nepal	*Rato masyang*	Included in OBN in 2008
NPGR-05424	Dhankuta		*Masyam*	Included in OBN in 2008
LRGR-133	Palpa	High-hill, Western Nepal	*Jhilinge*	Included in OBN in 2008
NPGR-00199	Kabhre	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
LRGR-148	Kavre	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
NPGR-05404	Illam	Mid-hill, Eastern Nepal	*Masyam*	Non core collection evaluation
NPGR-00074	Arghakhanchi	High-hill, Western Nepal	*Khaire masyang*	Non core collection evaluation
LRGR-151	Kavre	Mid-hill, Central Nepal	*Masyang*	Non core collection evaluation
LRGR-123	Palpa	High-hill, Western Nepal	*Jhilinge*	Included in OBN in 2008
NPGR-05380	Lamjung	High-hill, Western Nepal	*Masyang*	Non core collection evaluation
LRGR-118	Gulmi	Mid-hill, Western Nepal	*Jhilinge*	Non core collection evaluation
NPGR-05383	Tanahun	Mid-hill, Western Nepal	*Masyang*	Non core collection evaluation
NPGR-05398	Illam	Mid-hill, Eastern Nepal	*Masyam*	Non core collection evaluation
NPGR-05372	Gorkha	High-hill, Western Nepal	*Masyang*	Included in OBN in 2008
NPGR-05399	Illam	Mid-hill, Eastern Nepal	*Masyang*	Non core collection evaluation
LRGR-146	Kaski	Mid-hill, Western Nepal	*Masyang*	Non core collection evaluation
NPGR-05409	Illam	Mid-hill, Eastern Nepal	*Masyang*	Non core collection evaluation
NPGR-05401	Illam	Mid-hill, Eastern Nepal	*Masyang*	Non core collection evaluation
LRGR-73	Pyuthan	High-hill, Western Nepal	*Raiyans*	Non core collection evaluation
LRGR-145	Kaski	Mid-hill, Western Nepal	*Masyang*	Non core collection evaluation
NPGR-05410	Dhankuta	Mid-hill, Eastern Nepal	*Masyam*	Non core collection evaluation
LRGR-159	Kaski	Mid-hill, Western Nepal	*Masyang*	Non core collection evaluation
LRGR-135	Palpa	High-hill, Western Nepal	*Masyang*	Non core collection evaluation
JCR-08-2	Ganjam	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-10	Rayagada	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-12	Koraput	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-15	Kandhamal	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-27	Kandhamal	Orrisa, India	*Dangar rani*	Observation nursery/mother trial

Accession number	Collection districts	Ecological classification	Local name	Remarks
JCR-08-30	Koraput	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-35	Ganjam	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-45	Phulbani	Orrisa, India	*Dangar rani*	Observation nursery/mother trial
JCR-08-53	Darjeling	West Bengal, India	*Gaimung*	Observation nursery/mother trial
JCR-07-20	Imphal	Manipur, India	*Shyamalee*	Observation nursery/mother trial
JR-1		Gujarat, India		Observation nursery/mother trial
RBHP-7	Mandi,	HP, India		Observation nursery/mother trial
RBHP-16	Mandi	HP, India		Observation nursery/mother trial
RBHP-26	Kangra	HP, India		Observation nursery/mother trial
RBHP-28	Kangra	HP, India		Observation nursery/mother trial
RBHP-32	Uttarakhand	HP, India		Observation nursery/mother trial
RBHP-42	Uttarakhand	HP, India		Observation nursery/mother trial
RBHP-52	Uttarakhand	HP, India		Observation nursery/mother trial
RBHP-60	Uttarakhand	HP, India		Observation nursery/mother trial
RBHP-65	Kangra	HP, India		Observation nursery/mother trial
RBHP-76	Uttarakhand	HP, India		Observation nursery/mother trial
Adzuki bean	Bangor	Unknown		Purchased from supermarket
Rice bean	Illam	Mid-hill, Eastern Nepal		Bold seeded cream seeds
Ricebean	Baitadi	Mid-hill, Western Nepal		Small seeded, called '*gurans*'

DNA extraction

Bulk DNAs of 15 seeds of each accession (genotype) were used, isolated using the Phytopure Genomic DNA extraction kit (Tepnel Sciences PLC, Manchester, UK) (Annex I). The concentration of the isolated DNA samples was determined by comparing with a known concentration of λDNA on a 0.8% (w/v) normal agarose gel in 1xTBE buffer (0.09 M Tris-borate and 0.5 M EDTA) at 80 volts for 90 minutes with ethidium bromide staining. The concentration of the DNA extract was adjusted to 4-5 ng/µl for SSR analysis.

SSR analysis

Thirty five out of 109 SSR primers of adzuki bean (Han *et al.,* 2005) earlier identified as polymorphic loci (Bajracharya *et al.,* 2008) were used to elucidate genetic distinctness and detect the marker diversity in ricebean germplasm (Table 2). Amplification was in 20 µl containing 4-5 ng of genomic DNA, 10 µl of Reddy Mix™ PCR Master Mix (containing 3.0 mM $MgCl_2$; 10xPCR buffer, Taq polymerase and blue dye, ABgene, Epsom, Surrey, UK) and 20 µM of forward and reverse primers (Annex II). The PCR (polymerase chain reaction) was carried out in a MJ Research PTC– 100™ Programmable Thermal Controller with hot bonnet (MJ Research, INC, Waltham, MA, USA). The thermal cycling was programmed as initial denaturation at 94°C for 2 min., followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 30 sec., elongation at 68°C for 30 sec., followed by further elongation at 68°C for 2 min., and finally held at 4°C (Wang *et al.,* 2004 and Somta *et al.,* 2006). Amplified PCR products were run in 2.5% high resolution Amresco SFR agarose gel (Anachem LTD, Luton, Bedfordshire, UK) at a constant 90 volts for 4 hrs, and banding patterns were visualized under the ultraviolet illumination of gelcam stained with ethidium bromide. PCR product sizes were separated and estimated by comparing with DNA size standards of a 100 bp ladder (Promega Ltd, Southhampton, UK) in the GeneTool software of a Biodoc gel analyzer (Minibis Pro, Biosyatematica). Diversity analysis of generated marker data for test samples was carried following the protocol developed by Wang *et al.,* 2004 and Somta *et al.,* 2006.

Table 2: List of polymorphic SSR primers of Adzuki bean screened

Primers	Linkage	Forward Primer (5'-3')	Reverse Primer (5'-3')	Motif
CEDG127	4	GGTTAGCATCTGAGCTTCTTCGTC	CTCCTCACTTGGTCTGAAACTC	(TG)3(AG)9
CEDG018	5	AGCGTGTTTGTGGTGATAGC	ACACAGGAACGAACAAACCC	(AG)32
CEDG150	10	GAAGGGAATGAAAATGAAACCC	GTTCAATCCATTCAGTCTCC	(AG)14
CEDG214	5	CACTCACTGCAAAGAGCAAC	CTACCTATCTGAGGGACAC	(AG)4 AA(AG)31
CEDAAG002	2	GCAGCAACGCACAGTTTCATGG	GCAAAACTTTTCACCGGTACGACC	(AAG)16
CEDG204	7	CCTTGGTTGGAGCAGCAGC	CACAGACACCCTCGCGATG	(AG)15
CEDG043	3	AGGATTGTGGTTGGTGCATG	ACTATTTCCAACCTGCTGGG	(AG)14
CEDG021	10	GCAGAATTTTAGCCACCGAG	AAAGGATGCGAGAGTGTAGC	(AG)26
CEDG084	3	ATCAACTGAGGAGCATCATCGA	CAACATTTCAACCTTGGGACAG	(AG)13
CEDG015	1	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC	(AG)27
CEDG026	2	TCAGCAATCACTCATGTGGG	TGGGACAAACCTCATGGTTG	(AG)26
CEDG073	8	CCCCGAAATTCCCCTACAC	AACACCCGCCTCTTTCTCC	(AG)24
CEDG007				
CEDG008	5	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC	(AG)26
CEDG286	2	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC	(AG)23
CEDG294	3	CACCTTCTTAATCTCTTCACC	GGGTTTCTCTTAATTCATTGAGTC	(AT)27(AG)15
CEDG232	9	GATGACCAAGGTAACGTG	GGACAGATCCAAAACGTG	(AG)16
CEDG071	8	GGTCCATTGAGACGGATCGAG	TCCCACCTCAGCGGAATCC	(AG)9
CEDG253	8	CACTTCCATGATGACTCACC	CACCCTTCTTTATCCTCTTCG	(AG)30
CEDG090	1	ATAAGTAGAAATTGGTTCAAATG	GGTTCGTTAAAGTAACTTTTAAT	(AG)28
CEDG044	11	TCAGCAACCTTGCATTGCAG	TTTCCCGTCACTCTTCTAGG	(GT)10 AT(AG)18
CEDG141	1	CCAGGCATCCATGATGACC	GAAGTTGTTGGTAATGGTTGCCTC	(AT)6(AG)13
CEDG178	1	CGGAAGAAGAACGCAGAGTG	GCATCAACAAGGACTTCTGC	(AG)10 G(AG)5
CEDG118	6	AACCCAACCAACCCTTGTGGTAAG	GCTGGAATCATAATACCGCCTTGT	(AG)21
CEDG154	4	GTCCTTGTTTTCCTCTCCATGG	CATCAGCTGTTCAACACCCTGTG	(AG)14
CEDG037	6	GAAGAAGAACCCTACCACAG	CACCAAAAACGTTCCCTCAG	(AG)16 AC(AG)8
CEDG195	6	GAGGGTCTCCACTTTTGAAACCC	GATACTAAGGCTTTCTCCACCCAC	(AG)11
CEDG134	10	CTCCGTGTTGAAACAATGACG	GGTCTTTCTGATCTACGAACTTG	(AG)11
CEDG104	11	TATGGCCCGAGCAAACCTTG	CCGTTCGGTCTTCGGTTGAA	(AG)13
CEDG050	2	GGCAGAATCGTACAAGTG	GTCAGATTCTCGCTTGCATG	(AG)12
CEDG087	1	CCTCCTTGAAATTCTCCTTGA	CCTCTTGTGAACCTTGGGACAG	(AG)10
CEDG033				
CEDG305	3	GCAGCTTCACATGCATAGTAC	GAACTTAACTTGGGTTGTCTGC	(AG)22
CEDG018	5	AGCGTGTTTGTGGTGATAGC	ACACAGGAACGAACAAACCC	(AG)32
CEDG204	1	CCTTGGTTGGAGCAGCAGC	CACAGACACCCTCGCGATG	(AG)15

Data analysis

Each SSR band was treated as a separate locus. SSR bands (*loci*) were scored as present (1) or absent (0) in each accession. Genetic diversity parameters were estimated as total number of alleles, polymorphic alleles, alleles per polymorphic locus, percentage of polymorphic loci (PPL) and percentage of polymorphic alleles. Polymorphic information content (PIC) and Shannon Weaver index (SW) of each SSR marker as estimates of diversity were calculated and average diversity compared among accessions.

PIC values, also known as Nei's gene diversity, were determined as described by Weir (1996) using the formula

$$PIC = 1 - \Sigma(p_j)^2$$

where p_j is the proportion of the population carrying the *j*th allele (Nei, 1973).

Likewise SW was calculated as

$$H_o = -\sum_{i=1}^{k} P_i \ln P_i ,$$

where H_o is the diversity value of a polymorphic allele (band), P_i is the frequency of SSR band in a specific group, and $\sum_{i=1}^{k} P_i$ is the sum of frequencies of polymorphic bands (alleles) (Whitkus *et al.,* 1998). The average diversity (H_a) was calculated as

$$H_a = 1/n\Sigma H_o,$$

where n is the number of groups. The proportion of diversity between groups ($H_t - H_g$), relative to total diversity (H_t) was measured to represent the relative degree of genetic differentiation between groups. H_g is the mean diversity of each of the groups, and H_t is the total diversity of the groups (Shannon, 1948).

Genetic similarity with Jaccard's coefficient and genetic distance was calculated by NTSYSpc software (version 2.2) to compute the efficiency of the selected polymorphic SSR markers in grouping the ricebean accessions. The similarity matrices were subjected to cluster analysis using UPGMA (unweighted pair group method with arithmetic averages) method and principal component analysis. The association between the similarity matrix and genetic distance matrix measured was determined by the Mantel test (Mantel, 1967) using the MXCOMP function of NTSYS (Rohlf, 1992).

Research findings

Polymorphism of microsatellites in ricebean landrace genotypes

Thirty-five SSR polymorphic primer pairs were analyzed on selective stratified number of Indian and Nepalese ricebean genotypes (Table 1), of which 28 to 35 gave amplified products. Out of 35, only 30 pairs of Adzuki bean SSR primers were successful in amplifying genomic DNA fragments of most of the Indian accessions, and only 28 were successful in the check samples. A total of 85 DNA fragments in SSR analysis were yielded across all the accessions under study including three check samples (Table 3). The difference in product sizes for most the primers found less with fewer base pairs (bp) among the accessions from Nepal, India and the check samples under study. The estimation of product size of the fragments in bp therefore could not be made although the 100 bp size standard was being used but the difference in size as bands was measured based to the distance travelled onto gel. Figure 2 shows an example of polymorphism with multiple bands revealed by CEDG073 among the studied ricebean genotypes of India and Nepal. The allelic diversity parameters number of polymorphic loci, allele numbers, percentage of polymorphic loci (PPL), percent age of polymorphic alleles (PPA), polymorphic information content (PIC) and Shannon Weaver Index (SW) were calculated and are listed in Tables 3 and 4.

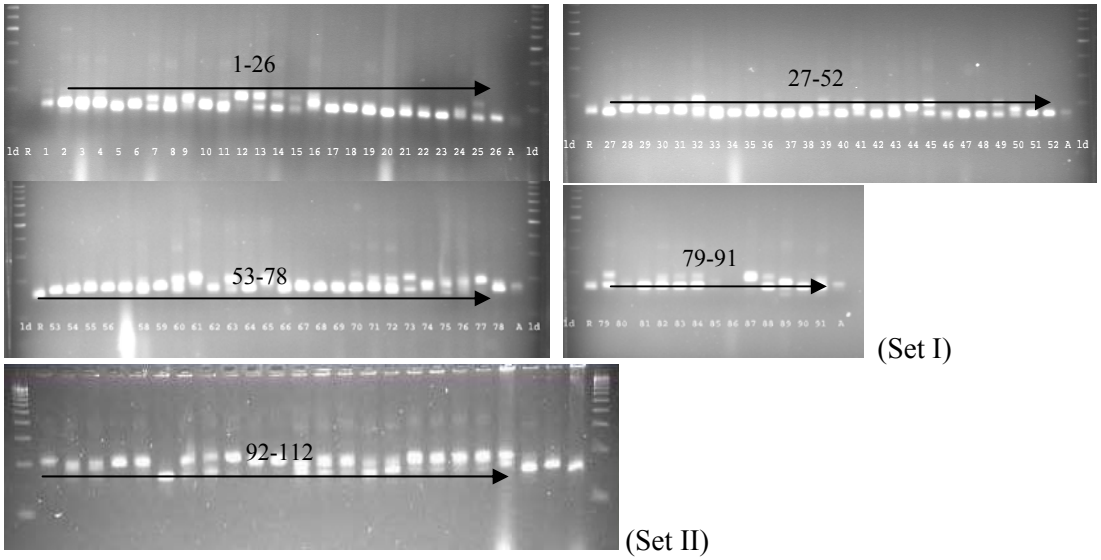


Figure 2*:* Portion of SSR banding patterns showing polymorphism produced by SSR primer CEDG073 in Nepalese and Indian ricebean genotypes. [From top to bottom: the first 4 gel photos(Set I) correspond to Nepalese landraces (numbered 1 to 91 in Table 1) and the last photo(Set II) to Indian landraces (21 accessions from 92 to 112 in Table 1). Each gel comprises the check samples and ladders.]

Allelic diversity of Nepalese genotypes

Thirty SSR primer pairs were found to be polymorphic and produced multiple fragments (as bands in gel) with 2-4 alleles per locus, while five primer pairs produced a single fragment (allele) in 91 Nepalese genotypes. These were CEDG008, CEDG018, CEDG104, CEDG127 and CEDG150. Therefore 86% of the loci produced polymorphism among the 91 Nepalese genotypes, and produced 75 (94%) polymorphic alleles (Table 3). Allele number thus varied from 2 to 4, with an average of 2.5 alleles per polymorphic locus. Similarly, the average number of amplified bands per primer was 2.3, with the highest number of alleles being 4 with primer CEDG033 (Table 4). The percentage of polymorphism (PIC) ranged from 3% (CEDG071) to a maximum of 63% (CEDG044), with an average of 24%: nine primers displayed higher than average polymorphism (Table 5). The highest Shannon Weaver diversity index (SW) was 1.18 (CEDG044) and the lowest 0.08 (CEDG071), with an average of 0.47 (Table 5).

Allelic diversity of Indian genotypes

Twenty-two of the 30 amplified primer pairs (73%) produced polymorphism among the 21 Indian genotypes, and 47 alleles (87%) were polymorphic with an average of 2.1 alleles per polymorphic locus, a comparatively low value. Eight primers (CEDG021, CEDG026, CEDG029, CEDG037, CEDG050, CEDG118, CEDG134 and CEDG232) were monomorphic, and amplified a single fragment (band). The number of alleles per polymorphic locus varied from 2 to 4, with the highest number in CEDG073 . The average number of amplified bands was 1.8, and PIC ranged from 9% (CEDG015, CEDG043, CEDG195, CEDG253, CEDG292 and CEDG294) to 71% (CEDG141) with an average of 27% polymorphism. Ten of the 22 primers showed polymorphism above the average PIC value. The SW index ranged from 0.19 to 1.38, with an average of 0.48 (Table 5).

Table 3*:* Summary of SSR diversity values calculated for ricebean accessions and all samples

Diversity parameters	Ricebean germplasm			
	Nepal	India	Checks	All accessions
Total sample analysed	91	21	3	115
Total SSR markers analysed	35	35	35	35
Total SSR markers amplified	35	30	28	30
Total polymorphic loci	30	22	21	30
% of polymorphic loci (PPL)	86	73	75	86
Total number of alleles	79	55	54	85
Alleles per locus	2.3	1.8	1.9	2.4
Total polymorphic alleles	75	47	47	80
Alleles per polymorphic locus	2.5	2.1	2.2	2.7
% of polymorphic alleles (PPA)	94	85	87	94
PIC (gene diversity, H')	0.25	0.27	0.50	0.32
Shannon Weaver Index (SW)	0.47§	0.48§	0.75	0.59β
Genetic differentiation ($H_t - H_g$)	0.12	0.11		

§=mean diversity of group of genotypes; β=total diversity of all groups of genotypes

Comparison of polymorphism among the genotypes of India and Nepal

The allelic polymorphism revealed by SSR primers in the Nepalese and Indian groups of genotypes was compared, both between the groups and also relative to the whole 115 accessions being studied. All 35 SSR primers amplified fragments across the genotypes, and yielded 85 fragments with an average of 2.4 alleles per locus, 32% PIC, and 0.59 of the total diversity scored as SW index (Table 3). The number of polymorphic alleles in each group was 75 in the Nepalese germplasm, 47 in the Indian germplasm and check samples, and 80 in all accessions. Polymorphism in the Indian germplasm was lower than in the Nepalese, possibly due to the small number of accessions. However, diversity values showed no significant variation, and both groups of germplasm were equally diverse with averages of 0.25 and 0.27 respectively. This may have resulted from the uneven distribution and frequency of the fragments (allelic unevenness) that occurred in the Indian accessions. This idea is further supported by the proportions of genetic differentiation, which did not differ (0.11 to 0.12) between the two sets of genotypes (Table 3).

Table 4: Allelic variation for 13 informative SSR loci in three collections of ricebean genotypes

Primers	Sequence Repeats	Number of alleles observed in ricebean genotypes at each of most informative SSR loci			
		Nepalese genotypes (n = 91)	Indian genotypes (n = 21)	Check populations (n = 3)	Total genotypes (n = 115)
CEDG073	(AG)24	2	4	1	3
CEDG015	(AG)27	3	2	-	3
CEDG033		4	2	3	4
CEDG090	(AG)28	3	2	2	3
CEDG071	(AG)9	2	2	2	4
CEDG044	(GT)10AT(AG)18	3	2	1	3
CEDG087	(AG)10	2	2	1	2
CEDG007		2	2	2	2
CEDG141	(AT)6(AG)13	2	3	2	4
CEDG204	(AG)15	3	2	2	3
CEDG178	(AG)10G(AG)15	2	2	2	2
CEDG214	(AG)4AA(AG)31	3	2	3	3
CEDAAG002	(AAG)16	2	2	2	2
CEDG305	(AG)22	3	2	2	3
Total polymorphic alleles		75	47	47	80
Average*		2.5	2.1	2.2	2.7

*= average no of alleles per locus calculated on total alleles generated by all polymorphic SSR loci.

All polymorphic SSR primers which produced clear polymorphic fragments in ricebean genomic DNA were predominantly dinucleotide sequences, mostly (AG) repeats except for CEDG044 and CEDAAG002 which had (GT)AT(AG) and (AAG) repeats (Table 2 and 3). The $(AG)_n$ motifs were predominant in the ricebean genome (except in the linkage of 4, 5 and 7) and were also found accompanied with (AT) in CEDG141, and with GT in CEDG044, as compound sequence repeats (Table 3). The number of (AG) repeats ranged from 9 to 32 (Table 2), showing that the SSR primers with (AG) repeats are useful in detecting the allelic polymorphism in genomic DNAs of ricebean.

Table 5: PIC (gene diversity) and SW (Shannon Weaver) indices for polymorphic SSR markers observed for group of the accessions of India and Nepal

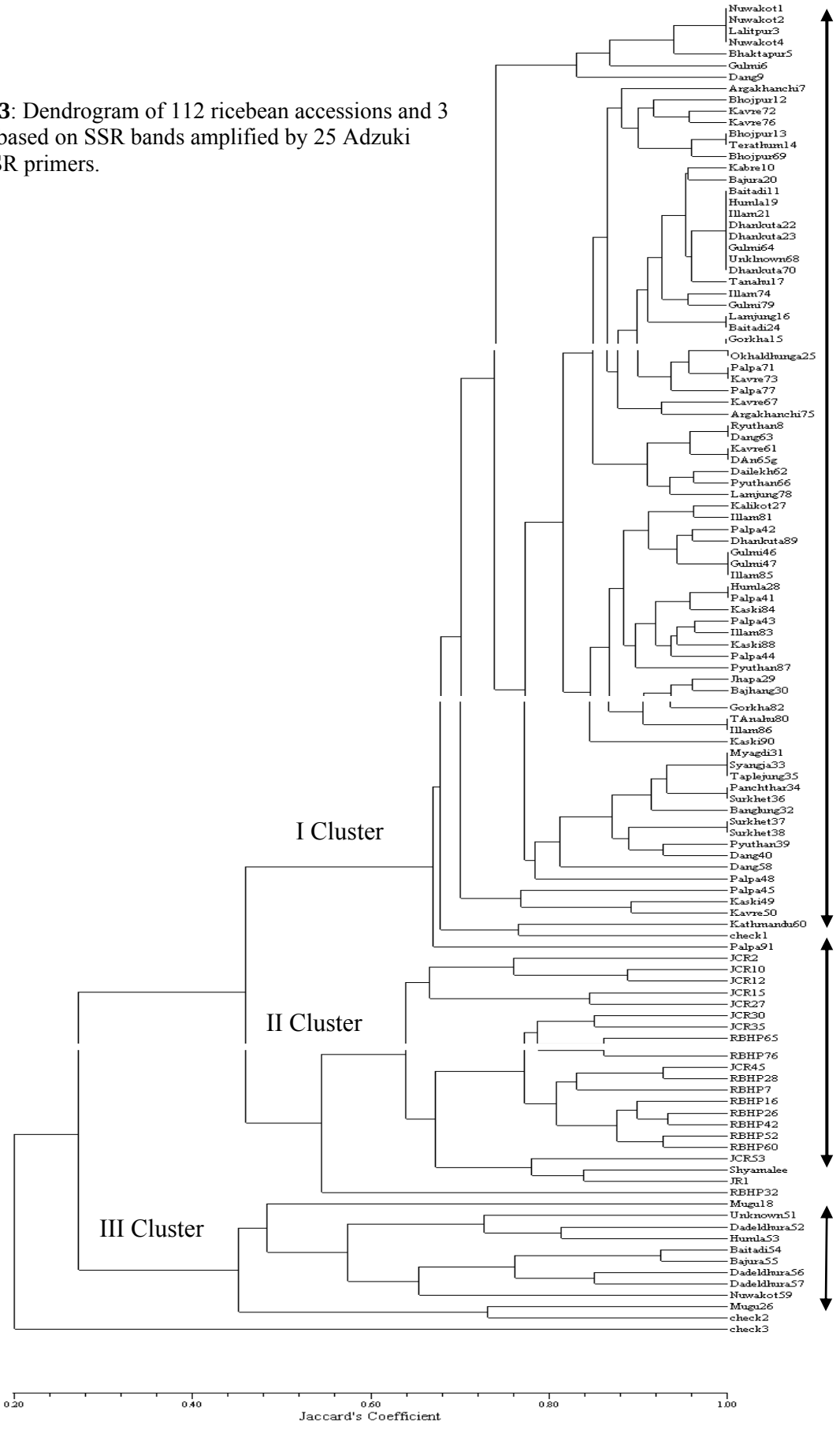
Primers	Linkage groups	PIC values		SW index	
		Nepalese accessions (n = 91)	Indian accessions (n = 21)	Nepalese accessions (n = 91)	Indian accessions (n = 21)
CEDG073	8	0.51	0.66	0.84	1.30
CEDG015	1	0.45	0.09	0.84	0.19
CEDG033	4	0.50	0.38	1.06	0.67
CEDG090	1	0.55	0.31	0.94	0.49
CEDG037	6	0.22		0.37	
CEDG043	3	0.11	0.09	0.22	0.19
CEDG029	2	0.23		0.54	
CEDG232	9	0.23		0.39	
CEDG253	8	0.08	0.09	0.18	0.19
CEDG286	2	0.18	0.10	0.36	0.21
CEDG292	4	0.24	0.09	0.47	0.21
CEDG294	3	0.05	0.09	0.12	0.19
CEDG071	8	0.03	0.44	0.08	0.63
CEDG050	2	0.19		0.38	
CEDG044	11	0.63	0.47	1.15	0.66
CEDG084	3	0.20	0.10	0.35	0.21
CEDG021	10	0.22		0.37	
CEDG118	6	0.21		0.37	
CEDG007		0.20	0.31	0.37	0.49
CEDG141	1	0.15	0.71	0.33	1.38
CEDG195	6	0.23	0.09	0.47	0.19
CEDG204	7	0.28	0.10	0.49	0.20
CEDG026	2	0.21		0.43	
CEDG154	4	0.07	0.18	0.17	0.38
CEDG178	1	0.46	0.09	0.65	0.19
CEDG214	5	0.22	0.49	0.44	0.68
CEDAAG002	2	0.31	0.61	0.55	1.02
CEDG134	10	0.18		0.32	
CEDG087	1	0.07	0.35	0.15	0.53
CEDG305	3	0.30	0.19	0.70	0.39
Average		0.25	0.27	0.47	0.48

Genetic diversity and relationships in Ricebean genotypes

Cluster Analysis

Pairwise Jaccard's coefficient for genetic similarities was calculated on the allelic data (Jaccard, 1908; Nei, 1972) generated by the 25 most informative loci across all genotypes. Cluster and principal component analyses using the unweighted paired group method with arithmetic averages (UPGMA) and correlation respectively depicted the distribution and relationships of the accessions as dendrogram and scatter plots (Figures 3 and 4). Jaccard's similarity coefficient ranged from 0.20 to 1.00 (Figure 3). At 68% level of similarity, the UPGMA dendrogram was clearly divided into three major genotype clusters (Figure 3).

Figure 3: Dendrogram of 112 ricebean accessions and 3 checks based on SSR bands amplified by 25 Adzuki bean SSR primers.



Cluster I corresponds to most of the Nepalese genotypes irrespective of their collection districts and agro-ecosystems, and cluster II to the Indian genotypes. Cluster III corresponds to the most diverse Nepalese genotypes, landraces from the high hills of Far West Nepal (except one accession from Nuwakot59), and were close to the check samples of adzuki bean and *Gurans*, which is similar to adzuki bean (checks 2 and 3 respectively) (Figure 3 and Table 2). These genotypes shared an average of 60% similarity in the dendrogram. In cluster II, the accessions from India, it is obvious that the JCR groups (collected from Orrisa) form a separate sub-cluster; RBHP groups (collected from Palampur) form another, and JR-1 (an accession from Gujrat), JCR-08-53 and Shyamalee (from West Bengal and Manipur) form a third. However, RBHP 32, RBHP 65, RBHP 76 and JCR 45 did not conform to this pattern. RBHP 65 and RHBP 76 clustered with the JCR group of accessions from Orissa, while JCR 45 clustered with the RBHP group. A large group (81 accessions) in cluster I grouped together and shared above 80% of similarity to each other in dendrogram (Figure 3). One of the three check samples (ricebean- bold grained) also fell in this cluster. These primers thus exhibited genetic relationships at DNA level among tested Nepalese and Indian ricebean genotypes, and also between Adzuki bean and ricebean.

Principal components analysis

The results of the principal components analysis (PCA) were compared with the cluster analysis. The first two components explained 46.3% of the total variation. The distribution and association of genotypes in both cluster analysis and PCA agreed well and the Nepalese genotypes were found to be more diverse than the Indian (Figure 4). Accessions NPGR-05384, NPGR-06591 (Mugu); NPGR-5435; NPGR-06725 (Humla); NPGR-00014 (Nuwakot); LRGR-3, LRGR-7, LRGR-8 (Dadeldhura); LRGR-55 (Baitadi) and LRGR-30 (Bajura) appeared distinct, and were dispersed in the scatter plot. These were all accessions collected from the high-hill districts of the Far West of Nepal except NPGR-00014, which was from central part of the country. These genotypes showed a similar level of diversity in the dendrogram (Figure 3).

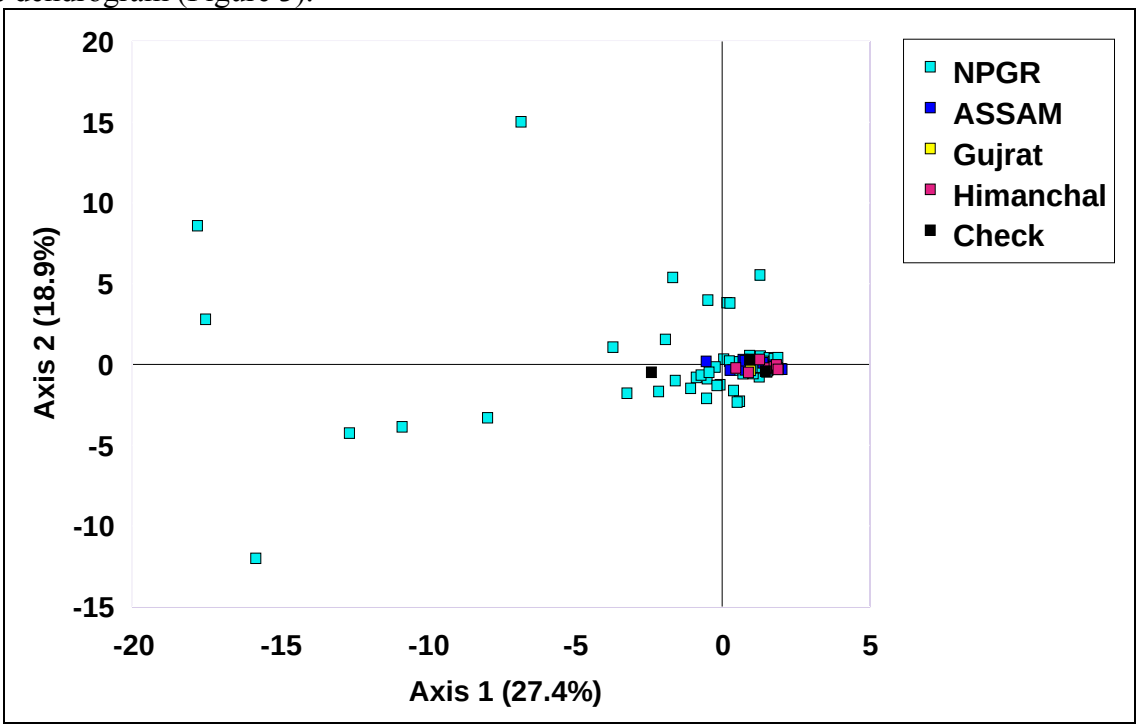


Figure 4: Scatter plot of Nepalese and Indian ricebean accessions, showing diversity based on PCA on 69 alleles generated by 25 primers

Discussion

The SSR technique has been used in characterisation and genetic relationship studies in genus *Vigna* (Wang *et al,* 2004, Han *et al.,* 2005, Dikshit *et al.,* 2007) and in several other crops. It is an efficient assay and has shown high levels of polymorphism in many crops including *Vigna* species (Yu *et al.,* 1999). SSR are an abundant resource in the genome, so are frequently used as markers in genetic studies. They have provided detailed information on genetic structures and gene flow in plant population studies, and are codominant (Powell *et al.,* 1996; McCouch *et al.,* 1997; Bonin *et al.,* 2001). Therefore, we employed SSR markers to detect diversity in Nepalese and Indian landrace populations. The DNA fragments amplified by adzuki bean SSR primers showed the availability of SSR markers in the ricebean genome that could be used for the genetic analysis and description of relationships in ricebean.

Given the paucity of research on molecular aspects of ricebean, characterisation and assessment of its diversity could have great significance in designing breeding and germplasm conservation strategies (Singh *et al.,* 2003). This is the first attempt to carry out systematic studies on molecular marker diversity on Nepalese ricebean germplasm. However, a few collections of cultivated ricebean have undergone morphological and molecular analysis (Seehalak, 2006). The selected polymorphic adzuki bean SSR markers enabled the detection of polymorphism in ricebean. Although they explained a lower diversity among the accessions, they distinguished the various genotypes, providing support for the use of SSR as a powerful tool to distinguish closely related individuals.

The evaluation of allelic polymorphism and resulting dendrogram revealed a level of diversity, which differentiated the genotypes into clusters of different sizes. This enabled us to identify variation in plant and seed morphology (to be determined in D3.3) and assisted in identification at the species level, because ricebean could be distinguished from the check sample of adzuki bean, a related species belonging to the *Angulares* section of sub-genus *Ceratropis*. The adzuki bean SSR primers used showed association between the nucleotide repeats and the level of polymorphism in ricebean. Primers with (AG) and (AAG) repeats were predominant in ricebean, and exhibited a high level of polymorphism. These were CEDAAG002, CEDG007, CEDG015, CEDG033, CEDG044, CEDG071, CEDG073, CEDG087, CEDG090, CEDG141, CEDG178, CEDG204, and CEDG305.

Conclusion

Thirty five adzuki bean SSR primers were used to detect genetic structure and relationships among Nepalese and Indian ricebean landraces. Of the primers evaluated, only 22-30 were found to be informative, with an average PIC of 0.25-0.27% in 91 Nepalese and 21 Indian genotypes respectively. These microsatellites were distributed across the ricebean genome and constituted di-nucleotides of AG. A dendrogram, and a PCA based on microsatellite polymorphism were consistent and showed genetic relationships among the groups of genotypes. Indian and Nepalese genotypes were equally diverse, and most could be genetically distinguished and described by SSR molecular marker traits. This molecular marker information on ricebean germplasm is of significance for any future research related to conservation of genetic resources and genetic enhancement of ricebean.

Acknowledgement

We express our sincere thanks to Drs. S.B. Neog, M.K. Modi, S.P. Singh (Department of Agricultural Biotechnology) and R.N. Sharma (Department of Plant Breeding and Genetics), all of Assam Agricultural University, Jorhat, for germplasm and laboratory facilities for

carrying out the molecular analysis of the Indian genotypes. We also thank Drs. N. Kumar (CSKHPKV, Palampur) and J.P. Yadavendra (GVT, Gujarat) for the ricebean germplasm provided for study and Dr K.D. Joshi for facilitating the transport of molecular consumables to India. Special thanks are due to the farming communities of India and Nepal for their support and provision of seed for this work.

References

Ajibade SR, Weeden NF & Chite SM (2000). Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. *Euphytica* **111**: 47-55.

Arora RK, Chandel KPS, Joshi BS & Pant KC (1980). Ricebean: tribal pulse of Eastern India. *Economic Botany* **34**: 260-263.

Bajracharya J, Singh S, Dangol B, Hollington PA & Witcombe JR (2008). Food security through ricebean research in India and Nepal (FOSRIN). *Report 2: Identification of polymorphic markers* (Ed PA Hollington). Khumaltar, Nepal: Nepal Agricultural Research Council and Bangor, Wales, UK: CAZS Natural Resources, College of Natural Sciences, Bangor University.

Bonnin I, Ronfort J, Wozniak F & Olivieri I (2001). Spatial effects and rare outcrossing events in *Medicago truncatula* (Fabaceae). *Mol Ecol* **10**: 1371-1383.

Brown SM, Hopkins MS, Mitchell SE, Senior ML, Wang TY, Duncan RR, Gonzales-Candelas F & Kresovich S (1996). Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in [*Sorghum bicolor* (L.) Moench]. *Theor. Appl. Genet*. **93**: 193-198.

Chaitieng B, Kaga A, Tomooka N, Isemura T, Kuroda Y, & Vaughan DA (2006). Development of black gram [*Vigna mungo* (**L**.) Hepper] linkage map and its comparison with an adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] linkage map. *Theor Appl Genet* **113**: 1261-1269.

Chandel KPS, Arora RK & Pant KC (1988). *Ricebean – a potential grain legume*. NBPGR Scientific Monograph No**. 12.** New Delhi, India. NBPGR.

Chaudhuri AP & Prasad B (1972). Flowering behaviour and yield of ricebean (*Phaseolus calacaratus* Roxb.) in relation to date of sowing. *Indian Journal of Agricultural Science* **42**: 627-630.

Dikshit HK, Jhang T, Singh NK, Koundal KR, Bansal KC, Chandra N, Tickoo JL & Sharma TR (2007). Genetic differentiation of Vigna species by RAPD, URP and SSR markers. *Biologia Plantarum* **3**: 451-457.

Doi K, Kaga A, Tomooka N & Vaughan DA (2002). Molecular phylogeny of *Vigna* subgenus *Ceratotropis* based on rDNA ITS and atpB-rbcL intergenic spacer of cpDNA sequences. *Genetica* **114**: 129-145.

Gautam R, Kumar N, Yadvendra JP, Neog SB, Thakur S, Khanal A, Bhandari B, Joshi KD & Hollington PA (2007). Food security through ricebean research in India and Nepal (FOSRIN). *Report 1: Distribution of ricebean in India and Nepal* (Ed PA Hollington)**.** Pokhara, Nepal: Local Initiatives for Biodiversity, Research and Development and Bangor, Wales, UK: CAZS Natural Resources, College of Natural Sciences, Bangor University.

Gupta S, Kozak m, Sahay G, Durrai AA, Mitra J, Verma MR, Pattnayak A, Thongbam PD and Das A (2009). Genetic parameters of selection and stability and indication of divergent parents for hybridization in ricebean (*Vigna umbellata* Thunb. (Ohwi and Ohashi)) in India. *Journal of Agricultural Science* **147**:581-588.

Han OK, Kaga A, Isemura T, Wang XW, Tomooka N & Vaughan DA (2005). A genetic linkage map for adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] *Theor Appl Genet* **111**: 1278-1287.

Jaccard P (1908) Nouvelle researches sur la distribution florale. *Bull. Soc. Vaud Sci Nat* **44**: 223-270

Kaga A (1996). *Construction and application of linkage maps for adzuki bean* (*Vigna angularis*). Doctoral dissertation, Kobe University, Japan.

Kaga A, Vaughan DA, & Tomooka N (2005). Molecular markers in *Vigna* improvement: understanding and using gene pools. In Lotz H, Wenzel G (eds) *Biotechnology in agriculture and forestry*, vol 55. Molecular marker systems . Springer, Berlin, Heidelberg New York, pp 171-187.

Lawn RJ (1979). Agronomic studies of Vigna species in South east Queensland. I Phenological response of cultivars in different sowing dates. *Aust. Jour. Agric. Res*. **30**: 855-870.

Li YC, Rodder MC, Fuhima T, Kirzhiner VM, Beiles A, Korol AB, & Nevo E (2000). Natural selection causing microsatellite diversgence in wild emmer wheat at the ecologically variable microsite at Ammiad, Isarel. *Theor. Appl. Genet*. **100**: 985-999.

Lohani S (1979). *Agricultural crops of Nepal* **1**: 282-285.

Lokesh PKV, Gupta SN & Behl RK (2003). Path. Coefficient analysis for various morphological and quality parameters in ricebean (*Vigna umbellata* Thunb. (Ohwi and Ohashi)). *National Journal of Plant Improvement* **5**: 116-119.

Lumpkin TA & McClary DC (1994). *Adzuki bean: Botany, production and uses*. CAB International, Wallingford, UK.

McCouch SR, Chen X, Paunad O, Temnykh S, Xu Y, Cho YG, Hang N, Ishii T, & Blair M (1997). Microsatellite marker development, mapping, and applications in rice genetics and breeding. *Plant Mol Biol* **35**: 89-99.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research.* **27**: 209-220

Nei, M. (1973). Analysis of gene diversity in subdivided populationa. *Proc. Natl. Acad. Sci*.. USA **70**: 3321-3323.

Powell W, Machary GC & Provan J (1996). Polymorphism revealed by simple sequence repeats. *Trends Plant Sci*. **1**: 215-222.

Rohlf, FJ (1992). *NTSYS-pc Numerical Taxonomy and Multivariate Analysis System Version 1.70*. State University of New York, Stony Brook, NY.

Sarma BK, Singh M, Gupta HS, Singh G & Srivastav L (1995). *Studies in ricebean germplasm*. Research Bulletin 34. Barapani (Umiam), India: ICAR research Complex for North East Hill Regions. Pp. 1-67.

Sarma BK, Das A and Bujarbaruah KM (2003). *Underutilized life support crop species: Production and research in North Eastern Hill Region of India*. Umiam, Meghalaya: ICAR Research Complex for NEH Region.

Seehalak W, Tomooka N, Waranyuwat A, Thipyapong P, Laosuwan P, Kaga A, & Vaughan DA (2006). Genetic diversity of the *Vigna* germplasm from Thailand and neighbouring regions revealed by AFLP analysis. *Gen Resources Crop Evol*. Doi 10.1007/s10722-004-7939-7

Shannon, CE (1948). A mathematical theory of communication. *Bell System Technology Journal*. **27**:379-423, 623-656.

Singh KP, Sareen M, Ashwini PK & Kumar A (2003). Interspecific hybridization studies in *Vigna radiata* (L) Wilczek and *Vigna umbellata*. *Nat. J. Plant Improv.* **3**:16-18..

Somta P, Kaga A, Tomooka N, Kashiwara K, Isemura T, Chaitieng B, Srinives P & Vaughan DA (2006). Development of an interspecific *Vigna* linkage map between *Vigna umbellata* (Thunb.) Ohwi and Ohashi and *V. nakashimae*) Ohwi) Ohwi and Ohashi and its use in analysis of bruchid resistance and comparative genomics. *Plant breeding* **125**: 77-84.

Souframanien J & Gopalkrishna T (2004). A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. *Theor Appl Genet* **109**: 1687-1693.

Tateishi Y (1985). A Revision of the Adzuki Bean Group, the Subgenus Ceratrotropis of the Genus (Vigna (Leguminosae). PhD thesis, Tohoku University, Japan.

Tomooka N, Lairungreang C, Nakeeraks P, Egawa Y & Thavarasook C (1991). Mung bean and the genetic resources. TARC, Japan.

Tomooka N, Vaughan DA, XU RQ & Doi K (1998). Wild relatives of crop conservation in Japan with a focus on *Vigna spp*: Introduction. Pp 45-61. In *Annual Report on Exploration and Introduction of Plant Genetic Resources*, **14**. Nat Inst Agrobiol Resources, Tsukuba, Japan.

Tomooka N, Kaga A, Vaughan DA & Jayasuriya AHM (2003). Advances in understanding the genus *Vigna* subgenus *Ceratotropis*. Pp 25-35. In AHM Jayasuriya and DA Vaughan (eds) Conservation and use of crop wild relatives. Proc Joint Dept Agric, Sri Lanka and Nat Inst Agrobiol Sci, Japan Workshop. Plant Genetic Resources Centre, Dept Agric, Sri Lanka.

Wang XW, Kaga A, Tomooka N & Vaughan DA (2004). The development of SSR markers by a new method in plants and their application to gene flow studies in adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] *Theor Appl Genet* **109**: 352-360.

Whitkus R, Cruz M de la, & Mota-Bravo L (1998). Genetic diversity and relationships of cacao (Theobroma cacao L.) in southern Mexico. *Theor. Appl. Genet*. **96**: 621-627.

Weir BS & Cockerham CC (1984) Estimating F statistics for the analysis of population structure. *Evolution* **38**: 1358-1370

Xu RQ, Tomooka N, & Vaughan DA (2000). AFLP markers for characterizing the Adzuki bean complex. *Crop Sci* **40**: 808-815.

Yamaguchi H (1992). Wild and weed adzuki beans in Japan. *Econ. Bot*. **46**: 384-394.

Yee E, Kidwell KK, Sills GR & Lumpkin TA (1999). Diversity among selected *Vigna angularis* (adzuki) accessions on the basis of RAPD and AFLP markers. *Crop Sci* **39**: 268-275.

Yu K, Park SJ, & Poysa V (1999). Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). Genome **42**: 27-34.

Annex I

DNA Extraction Procedure for bulk samples

1. Grind 15 seeds of ricebean of each sample to a fine powder in a coffee-bean grinder and discard it to minimise cross contamination between samples.
2. Collect the fine powder in a labelled polythene bag.
3. Dry the grinder with pressurised air to remove all dust, swab it with ethanol and make sure it to dry before the next sample.
4. Weigh approx 1 g (±0.05 g) of ground ricebean into sterile 15 ml centrifuge tubes labelled A and B to identify duplicates.
5. Add 4.0 ml of Reagent 1 from the Nucleon Phytopure Plant DNA Extraction kit.
6. Add 1.4 ml of Reagent 2 from the Nucleon Phytopur Plant DNA Extraction kit.
7. Vortex thoroughly to mix approx 15 seconds.
8. Incubate at 65^{o}C in a controlled-temperature water bath for 10 mins. Then cool on ice for 20 mins.
9. Transfer 1.8 ml from the top to a 2 ml micro-centrifuge tube and centrifuge at 13000 g for 15 min at 4^{o}C.
10. Transfer 0.5 ml of the supernatant to a fresh micro-centrifuge tube and place on ice for 20 mins.
11. Add 0.5 ml of chloroform (stored at -20^{o}C).
12. Add 0.1 ml of resuspended Nucleon Phytopure DNA Extraction resin.
13. Vortex for 16 mins.
14. Place on a rotating platform shaker for 10 mins. At room temperature to mix.
15. Centrifuge at 16000 g for 15 mins.
16. Transfer 0.5 ml of the upper phase (containing DNA) to a fresh micro-centrifuge tube.
17. Add 0.5 ml of propan-2-ol (stored at -20^{o}C) and gently invert tube 10 times to mix.
18. Centrifuge at 16000 g for 15 min to pellet DNA.
19. Discard supernatant and add 0.5 ml 70 % (v/v) ethanol to pellet.
20. Centrifuge at 16000 g for 5 mins at room temperature to pellet DNADecant supernatant and air dry pellet for 10 mins.
21. Resuspend DNA in 0.5 ml TE buffer and allow to rehydrate for 1 hour at room temperature
22. Store DNA at 4^{o}C for upto 1 month or at -20^{o}C for more than a month in the labelled micro-centrifuge tube.

Annex II

Microsatellite protocol (Li et al, 2000) with some modifications in $MgCl_2$

DNA dilution:

5 µl of CTAB DNA extract (50 ng of template DNA)
95 µl of sterilized distilled water (SDW)

Primer mix:
0.1µl of 20 µm of each primer
1.3 µl of SDW

Each of 15 µl PCR Master mix contained:
7.5 µl of ABgene master mix (10x Taq buffer, 1.5-3.0 mM $MgCl_2$, 50 mM KCl, pH 8.5; 20 µm
dNTPs mix)
6 µl of DNA dilution aliquot
1.5 µl of primer mix aliquot

Thermal cycling

After everything is aliquoted into thermocycler tubes (PCR plates), tubes were capped and placed in MJ Research thermocycler for amplification using the following thermal cycling PCR profile:

A PCR profile of initial denaturation at 94oC for 2 min followed by 35 cycles of 94^{o}C for 30 sec, 50^{o}C for 30 sec and 68^{o}C for 30 sec with initial denaturation at 94^{o}C for 2 min and final hold at 4^{o}C for infinite till to run electrophoresis was also tested to have a good amplification product.