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Report 2: Identification of polymorphic markers in ricebean

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Report on the identification of polymorphic markers in ricebean

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Executive summary

This document, Deliverable 3.1 of the FOSRIN (Food Security through Ricebean Research in India and Nepal) project, documents the identification of polymorphic markers in ricebean (*Vigna umbellata*). Ricebean is an underutilised grain legume grown mainly in marginal hill areas in Northern India and Nepal It belongs to the *Ceratropis* genus, section *Angulares*, and is believed to be a domesticated form of *V. umbellata* var. *gracilis*, a cross-fertile wild type native to Indo-China. It is closely related to adzuki bean, *V. angularis*. Although ricebean has high nutritional quality and plays an important role in food security and in the farming systems in the areas to which it is adapted, very little work has ever been done on the crop, and there has been no modern plant breeding, despite its very high phenotypic diversity.

A stratified sample of 27 ricebean genotypes from Nepal, sampled on diversity in local names, geographical origin, agro-environment, growth habit and seed traits, was selected to cover the range of diversity, together with a bold (large)-seeded check and an adzuki bean check. DNA was extracted using the modified CTAB method, and used for simple sequence repeats (SSR) analysis: a total of 109 primer pairs mapped on an existing adzuki bean linkage map, were used to identify primers detecting polymorphism in ricebean. Forty nine of these gave amplified products, with 93 DNA products detected. Of these, 35 SSRs were polymorphic, with 2-4 alleles per primer for the whole set including the adzuki bean check. Eleven of these primers contained AG repeats: one had AAG repeats and another GT(AT)AG repeats. They belonged to linkage groups 1, 2, 3, 6, 8, 10 and 11 of the ricebean genome, and explained the variation at the DNA level both between the ricebean accessions and between ricebean and adzuki bean. Although the ricebean accessions were diverse, they showed less diversity than had been expected.

Dendrograms were constructed based on UPGMA analysis to compare the groupings and identify the primers that best described the ricebean diversity. Three accession from the mid to high-hills in the far west of Nepal, which had indeterminate growth habit, clustered close to adzuki bean when grouped using eight polymorphic primers with more than three bands per primer. Two of these were also close when clustering used the 13 polymorphic primers with PIC greater than the average found.

The primers identified will be used in the next phase of the work, which is to assess molecular marker diversity in ricebean landraces selected for their good performance in the field trials held in 2006/07.

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1. INTRODUCTION

Ricebean [*Vigna umbellata* (Thunb) Ohwi and Ohashi] is an under-utilised grain legume cultivated in the hill areas of North, East and West India and of Nepal, often 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*, 2000a; Tomooka *et al* 2003). The original centre of domestication of the Asian *Vigna* species is thought to be Indo China, and they belong to the subgenus *Ceratotropis,* comprising 3 sections, *Ceratotropis*, *Aconitifoliae* and *Angulares*. *Angulares* is the most recent and diversified section of the subgenus (Doi *et al*, 2002), to which ricebean belongs. Ricebean is believed to be domesticated from a wild form *V. umbellata* var *gracilis* which is a cross fertile type. These wild types have been reported to occur in natural and disturbed habitats and are of indeterminate, photoperiod-sensitive, freely-branching, twining plant types with small seeds. Most ricebean landraces cultivated in Nepal are similar to these wild types.

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 from the east to the western limits of the country. It is an important food legume, particularly in the mid-hills of Nepal, and has a pivotal role as a pulse in supporting the food security of the rural poor people in this area. 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 to restore soil fertility by through biological nitrogen fixation (Lohani, 1979). However, despite these advantages it is little exploited, and most of its cultivation and production is for local consumption.

The multiple needs of farming households, their socioeconomic resources, and the prevalent agro-ecological conditions have greatly supported the conservation of crop diversity in Nepal, where ricebean landraces have been locally produced, maintained, and built up by farmers over generations. Normally, ricebean is not grown on the main cultivable land of a holding, but on marginal and fallow land, slopes and bunds under rainfed condition in maize-based cropping systems. However, in the lowlands it is also grown with rice in bunds. Ricebean is therefore most common between 700 and 1400 masl, but is also found between 300 and 600 masl and even to 2400 masl in Humla, a high-hill district. The crop is considerably neglected, and productivity is very low, averaging around 200-300 kg ha^{-1}.

1.1 Biosystematics of the *Vigna* genus

The genus *Vigna* is a large, heterogeneous genus distributed throughout the tropics which contains several important cultivated species including ricebean. Marechal *et al* (1978) developed a biosystematics' monograph of *Phaseolus-Vigna* complex showing phylogenetic relationships among the seven subgenera of African, Asian and American types. *Ceratotropis* is the Asian subgenus consisting of 17 species as wild and cultigens (Verdcourt, 1970). Tomooka *et al* (2000b), Tateishi & Maxted (2002) later found 4 new species and the number increased to 21. All these diploid species have a haploid chromosome number of 11 except *V. reflexo-pilosa,* which is tetraploid (2n=4x=44) (Chaitieng *et al,* 2006). These wild and domesticated species are collectively known as the Asian *Vigna* (Table 1.1).

Table 1.1: The eight cultivated species of the Asian *Vigna* of subgenus *Ceratotropis*

Species	Common name /local name	use	Taxa of wild form	Distribution of wild form
V. radiata	mungbean green gram	food	var. sublobata	E. Africa, Madagascar, Asia, New Guinea, Australia
V. mungo	black gram black matpe urd	food	var. silvestris	India, Myanmar
V. angularis	adzuki bean red bean	food	var. nipponensis	Himalayas, N. Myanmar, China, Korea, Japan
V. umbellata	**ricebean**	**Food, forage, green manure**	**as for cultigen**	**E. India, Myanmar, Thailand, Indo-China, China**
V. aconitifolia	moth bean mat bean	food cover crop	as for cultigen	Pakistan, India
V. trilobata or *V. stipulacea*	pillipsesara bean jungali bean	forage food cover crop	as for cultigen	India, Sri Lanka, Myanmar
V. reflexo-pilosa var. glabra = *V. glabrescens*	lentille de creole	forage food	var. reflexo-pilosa	S.E. Asia, S Japan
V. trinervia	tua pee	cover crop food	as for cultigen	Madagascar, India, Sri Lanka, Thailand, Malaysia, Indonesia, Papua New Guinea

Source: adapted from Tomooka *et al,* 2003

2.1 Genetic diversity in ricebean

The evaluation of genetic diversity with markers provides information on genetic relationships among the genotypes. The observed genetic variation and construction of linkage maps can then be efficiently used in breeding of new varieties with desired traits (Paterson *et al,* 1991). Variability studies in legumes are largely based on their morphology, agronomic behaviour, and biochemical traits with a considerably low level of polymorphism (Yamaguchi, 1992; Lumpkin & McClary, 1994). However, the Asian *Vigna* have been studied using a variety of molecular techniques. Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNAs (RAPDs), inter simple sequence repeats (ISSRs), and microsatellites or simple sequence repeats (SSRs) are the molecular marker techniques that have been extensively used in genome analysis.

Construction of linkage maps and phylogenetic relationships and genetic diversity studies have been carried out in cultivated and natural species of *Vigna* including subgenera C*eratotropis* (Han *et al,* 2005; Xu *et al,* 2000; Kaga, 1996; Tomooka *et al,* 1998; Souframanien & Gopalkrishna, 2004; Yee *et al,* 1999; Ajibade *et al,* 2000; 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 and Chaitieng *et al,* 2006) and a large number of SSR markers have been developed for adzuki bean (Wang *et al,* 2004 and Han *et al,* 2005). These SSR markers have been used in comparative linkage maps in other related legumes and have provided information on genetic relationships among the related species.

The National Grain Legume Research Programme (NGLRP) is one of the commodity programmes of the Nepal Agriculture Research Council (NARC), and undertakes breeding research to improve legume crops. Unfortunately, ricebean is not included in the national legume programme and therefore no systematic studies on its potential, diversity and use value have been initiated in Nepal. A collection of ricebean germplasm held by the NARC genebank contains a large number of landrace accessions collected by the Agriculture Botany Division (ABD) and the NARC Plant Genetic Resource Unit (PGRU) from different districts of the country in the early 1980s. These were obtained by different national and international collaborative collection and exploration missions. It has a collection of 149 accessions from 29 districts (ABD, 2004). In addition, 156 accessions were collected from 16 districts in Nepal in 2006 (Gautam *et al,* 2007) as part of the current project's activities. The collection information shows that in Nepal ricebean is mainly grown in the western hills.

Farmers classify these landraces according to days to maturity, seed colour and grain size, but no systematic study has been done to give a greater understanding of ricebean diversity and genetic structure, and there has been no plant breeding activity. However, the germplasm is thought to possess valuable diversity, which may be useful for breeding improved varieties with desired traits. As a result, one of the current project's activities as an agro-morphological and DNA-based molecular diversity study to analyse the genetic diversity of local ricebean germplasm. Agro-morphological characterization and evaluation of local ricebean germplasm, and an SSR diversity analysis, were carried out in field and laboratory conditions in 2007. The objective was to determine the agro-morphological diversity of the germplasm, to analyze the molecular marker data using SSRs, and to identify the polymorphic *loci* (markers). The primers for these *loci* will then be used to investigate the genetic variation of local ricebean germplasm that is under cultivation in different agro-ecosystems in Nepal This report (Project Deliverable 3.1) is on the identification of these polymorphic primers.

2. MATERIALS and METHODS

2.1 Plant materials

Table 2 lists the ricebean genotypes used for screening SSR primers for polymorphic *loci*. They were collected from different districts and agro-ecosystems in Nepal between 1976 and 2006, and are conserved in the national genebank by NARC at Khumaltar (Figure 2.1, Annex 2).

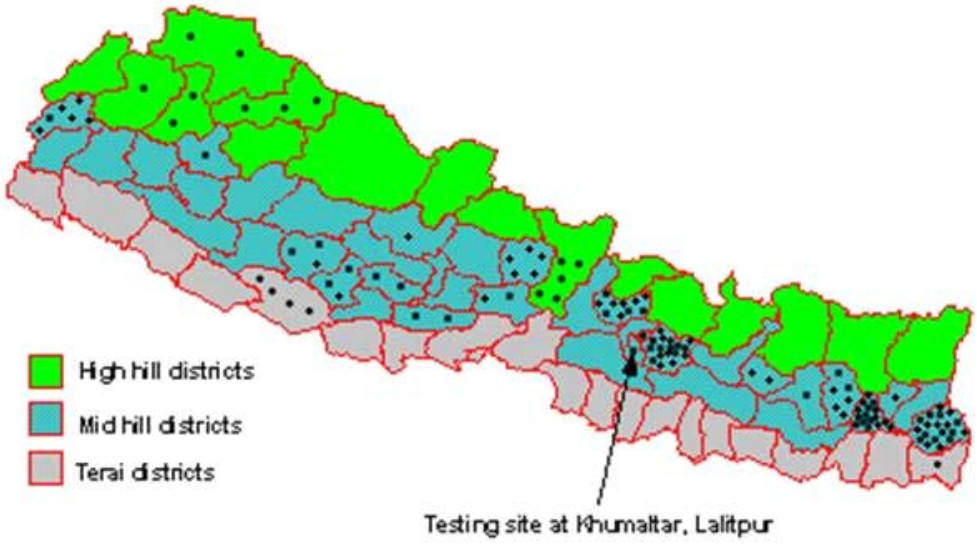


Figure 2.1: Ricebean germplasm and its distribution over agro-ecosystems and districts in Nepal

Three in the list (LRGR*nnn*) were collected by LI-BIRD during the implementation year (2006) of the current project. The whole set of 27 accessions were a stratified sample selected from the list of 120 NPGR and LRGR germplasm based on passport and agro-morphological diversity data. The sampling criteria employed were diversity in local names, collection districts representing the agro-ecological range, growth habit and seed characteristics. The set represented the range of diversity of ricebean germplasm, and would help in identifying the primers with high polymorphism. The accession number, seed colour, shape and size, growth habit (determinate and indeterminate), and the sites representing the collection districts and agro-ecological range and collection year of these stratified samples are presented in Table 2. One bold grained ricebean, collected from Ilam (Eastern Nepal), and an accession of adzuki bean were included as check samples.

Table 2.1: List of stratified accessions of ricebean used in screening of SSR primers for identification of polymorphic primers.

Accessions	Districts collected	Ecological classification	Year collected	Remarks
Ricebean (bold) (A)	Ilam	Check sample	2006	Collected by Dr P. Anderson
adzuki bean (B)	Unknown	Check sample	2007	Received from Dr P. A. Hollington, UK commercial sample
NPGR 00007	Nuwakot	Mid-hill, Central Nepal	1987	indeterminate, greenish yellow, cylindrical seeds
NPGR 00010	Lalitpur	Mid-hill, Central Nepal	1987	indeterminate, greenish yellow, cylindrical seeds
NPGR 00015	Bhaktapur	Mid-hill, Central Nepal		indeterminate, greenish yellow, cylindrical seeds
NPGR 00073	Gulmi	Mid-hill, Western Nepal	1988	indeterminate, mottled, drum shaped seeds
NPGR 00076	Arghakhanchi	High-hill, Western Nepal	1976	indeterminate, mottled, drum shaped seeds
NPGR 00087	Pyuthan	High-hill, Western Nepal	1993	indeterminate, greenish yellow, drum shaped seeds
NPGR 00090	Dang	Terai, Western Nepal		indeterminate, mottled , cylindrical seeds
NPGR 00194	Kavre	Mid-hill, Central Nepal	1987	indeterminate, greenish yellow, drum shaped seeds
NPGR 01975	Baitadi	Mid-hill, Western Nepal	1985	indeterminate, greenish yellow, cylindrical seeds
NPGR 05364	Bhojpur	High-hill, Eastern Nepal		indeterminate, greenish yellow, cylindrical seeds
NPGR 05370	Terhathum	High-hill, Eastern Nepal	1997	medium, red, cylindrical seeds
NPGR 05373	Gorkha	High-hill, Western Nepal	1987	indeterminate, greenish yellow, cylindrical seeds
NPGR 05377	Lamjung	High-hill, Western Nepal	1987	indeterminate, black, cylindrical seeds
NPGR 05382	Tanahu	Mid-hill, Western Nepal	1994	indeterminate, greenish yellow, cylindrical seeds
NPGR 05384	Mugu	High-hill, Western Nepal	1987	determinate, greenish yellow, globose seed
NPGR 06756	Humla	High-hill, Western Nepal	1988	determinate, greenish yellow, cylindrical seed
NPGR 05391	Bajura	Mid-hill, Western Nepal	1982	medium, greenish yellow, cylindrical seed
NPGR 05396	Illam	Mid-hill, Eastern Nepal	1997	indeterminate, greenish yellow, drum shaped seeds
NPGR 05420	Dhankuta	Mid-hill, Eastern Nepal	1987	indeterminate, red drum shaped seeds
NPGR 08380	Myagdi	Mid-hill, Western Nepal	1987	medium, mottled drum shaped
NPGR 07882	Bajhyang	High-hill, Western Nepal	1976	medium, red cylindrical seeds
NPGR 09391	Syangja	Mid-hill, Western Nepal	1994	indeterminate, mottled cylindrical seeds
NPGR 09461	Panchthar	High-hill, Eastern Nepal	1991	determinate, greenish yellow drum-shaped seeds
NPGR 09464	Taplejung	High-hill, Eastern Nepal	1994	indeterminate, greenish yellow cylindrical seeds
LRGR 042	Surkhet	Mid-hill, Western Nepal	2006	indeterminate, mottled cylindrical seeds
LRGR 025	Bajura	Mid-hill, Western Nepal	2006	determinate, light green, drum shaped seeds
LRGR 054	Baitadi	Mid-hill, Western Nepal	2006	determinate, greenish yellow, globose seed

2.2. DNA extraction

Five plants per accession were grown per pot (approx 3 litre sized) filled with sandy moistened soil in a greenhouse under natural daylength in April and May 2007. Pots were watered every day after emergence, but no fertilizer was applied. Genomic DNA was extracted from bulked young healthy leaves of five plants (aged about 3 weeks) of each accession using the modified CTAB method (Roger and Bendich, 1988). The concentration of the DNA samples was determined by comparing with a known concentration of λDNA on a 0.5% normal agarose gel in 1xTBE buffer (0.09 M Tris-borate and 0.5 M EDTA) at 80 volts for 90 min with ethidium bromide staining. The concentration of the DNA extract was adjusted to 4-5 ng/µl for SSR analysis.

2.3 SSR analysis

A total of 109 SSR primer pairs (Annexe 1), mapped on the adzuki bean linkage map developed by Han *et al,* (2005), were used to identify primers detecting polymorphism in ricebean germplasm. Amplification was performed in 20 µl volume 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. 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). This was programmed as follows: 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 to hold at 4°C for infinite (Wang *et al,* 2004 and Somta *et al,* 2006). Amplified products were run in 2.5% high resolution Amresco SFR agarose gel (Anachem LTD, Luton, Bedfordshire, UK) at constant voltage of 90 for 4 hrs and banding patterns were visualized by ultraviolet illumination of ethidium bromide stained gel under the Gelcam. Amplified PCR product sizes were estimated by comparing with DNA size standards in the GeneTool software of a Biodoc gel analyzer (Minibis Pro, Biosyatematica). A 100 bp ladder (Promega UK Ltd Southampton, UK) was used as a known standard size marker.

2.4 Data analysis

Each SSR band was treated as a separate *locus*. SSR bands (*loci*) were scored as present (1) and absent (0) in each accession. Genetic diversity was estimated as total number of alleles or of polymorphic alleles, polymorphic alleles per *locus*, percentage of polymorphic *loci*, and polymorphic information content (PIC) and Shannon Weaver index (SW) of each SSR marker. 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 jth allele (Nei, 1973; Shannon, 1948).

Genetic similarity by Jaccard's coefficient and cluster analysis using UPGMA (unweighted pair group method with arithmetic average) method was calculated with NTSYSpc software (version 2.2) to compute the efficiency of the selected polymorphic adzuki bean SSR markers screened in grouping the ricebean accessions (Rohlf, 1992).

3. Results

In this screening of polymorphic primers, the parameters for genetic variation observed for a total of 27 ricebean accessions consisting of different seed morphotypes and collections from different agro-ecosystems of the country are summarized in Tables 3.1 and 3.2. One hundred and nine adzuki bean

SSRs were analyzed, of which 49 gave amplified products. A total of 93 DNA fragments were detected in the accessions of ricebean and the adzuki bean check under the screening analysis of primers. Of these, 35 SSRs were polymorphic with 2-4 alleles per primer for the whole set of accessions including the adzuki bean checks. The difference in product sizes for most the primers was small, with less base pair (bp) diversity between adzuki bean and the ricebean accessions. However, an estimation of the product size of the accessions in bp could not be made, even although the 100 bp size standard was being used, probably because the product sizes were smaller than the standard size.

Table 3.1*:* Summary of SSR diversity values calculated for ricebean accessions and all samples

Diversity parameters	Ricebeans	All accessions including adzuki bean
Number of accessions considered	27	29
Number of SSR primers screened	109	109
Number of primers amplified	49	49
Number of monomorphic primers	19	14
Number of polymorphic primers	30	35
Percent of polymorphic primers	61	71
Total number of alleles (bands) observed	85	93
Number of alleles /primer	1.7	1.9
Total number of polymorphic alleles (bands)	63	79
Number of alleles/polymorphic primer	2.1	2.3
Percent of polymorphic alleles (bands)	74	85
Polymorphic information content (PIC)	0.26	0.24

Of the total of 85 bands amplified by 49 primers in the ricebean accessions (excluding adzuki bean), 63 (74%) were polymorphic with an average of 2.1 polymorphic bands per primer. This is close to the average value of 2.3 polymorphic bands per primer when computed for all accessions, including the check sample of adzuki bean (Table 3.1). Similarly, the average number of amplified bands per primer was 1.7 for the ricebean accessions, and 1.9 for all accessions including adzuki bean. The percentage of polymorphism as PIC ranged from 7 % (CEDG018) to a maximum of 67 % (CEDG073), with an average of 24 %. Likewise the Shannon diversity index value ranged from 1.28 (CEDG073) down to 0.17 (CEDG007, CEDG021 and CEDG141) with an average of 0.41 for the ricebean accessions (Table 3.2).

All those polymorphic primers with bold fonts (Table 3.2) contained AG repeats except for CEDAAG002, which had AAG repeats, and CEDG044 which contained GT(AT)AG repeats. These polymorphic primers belong to the linkage groups of 1, 2, 3, 6, 8, 10 and 11 of the ricebean genome. These primers explained the variation at DNA level among the ricebean accessions, and also between adzuki bean and the ricebean accessions.

Table 3.2: PIC and Shannon indices of each polymorphic adzuki bean SSR primer (Primers with PIC higher than the average value 0.24 of ricebean accessions are shown in bold)

Primers	Linkage	Alleles (bands)	PIC values		Shannon index	
			All accessions	Ricebean	All accessions	Ricebean
CEDG305	3	2	0.07		0.15	
CEDG127	4	2	0.22	0.17	0.38	0.30
CEDG018	5	2	0.07	0.07	0.16	0.16
CEDG150	10	2	0.26	0.22	0.38	0.38
CEDG214	5	2	0.19	0.11	0.34	0.22
CEDAAG002	**2**	**3**	**0.66**	**0.65**	**1.30**	**1.21**
CEDG292	4	2	0.14		0.27	
CEDG204	7	2	0.15	0.09	0.29	0.18
CEDG043	3	2	0.08	0.09	0.17	0.18
CEDG021	10	2	0.14	0.08	0.26	0.17
CEDG084	3	2	0.13	0.07	0.26	0.16
CEDG087	1	2	0.09		0.19	
CEDG033	4	2	0.09		0.18	
CEDG015	**1**	**3**	**0.48**	**0.43**	**0.79**	**0.62**

Table 3.2: PIC and Shannon indices of each polymorphic adzuki bean SSR primer (Primers with PIC higher than the average value 0.24 of ricebean accessions are shown in bold)

CEDG026	2	2	0.24	0.19	0.41	0.34
CEDG029	2	2	0.08		0.18	
CEDG073	**8**	**4**	**0.68**	**0.67**	**1.45**	**1.28**
CEDG007		2	0.20	0.08	0.35	0.17
CEDG008	5	2	0.16	0.09	0.30	0.19
CEDG286	**2**	**2**	**0.36**	**0.28**	**0.54**	**0.45**
CEDG294	**3**	**3**	**0.35**	**0.30**	**0.71**	**0.63**
CEDG232	**9**	**2**	**0.30**	**0.24**	**0.48**	**0.41**
CEDG071	8	2	0.15	0.16	0.28	0.30
CEDG253	**8**	**2**	**0.28**	**0.24**	**0.45**	**0.40**
CEDG090	1	3	0.14	0.08	0.32	0.17
CEDG044	**11**	**3**	**0.42**	**0.30**	**0.83**	**0.63**
CEDG141	1	2	0.14	0.08	0.27	0.17
CEDG178	**1**	**2**	**0.64**	**0.65**	**1.06**	**1.07**
CEDG118	6	2	0.16	0.09	0.30	0.19
CEDG154	4	2	0.17	0.10	0.31	0.58
CEDG037	**6**	**3**	**0.59**	**0.58**	**0.52**	**0.45**
CEDG195	**6**	**2**	**0.36**	**0.32**	**0.38**	**0.26**
CEDG134	**10**	**2**	**0.36**	**0.40**	**0.54**	**0.59**
CEDG104	11	2	0.27	0.23	0.38	0.30
CEDG050	**2**	**3**	**0.35**	**0.30**	**0.63**	**0.21**
Mean			**0.26**	**0.24**	**0.45**	**0.41**

Figure 3.1 illustrates the extent of polymorphism observed among ricebean, as revealed by CEDG150, CEDG118, CEDAAG002 and CEDG037. Although the ricebean accessions in the study were diverse, they showed a lower level of diversity than expected. Most of the amplified DNA fragments were close to adzuki bean fragments in size (Figure 3.1).

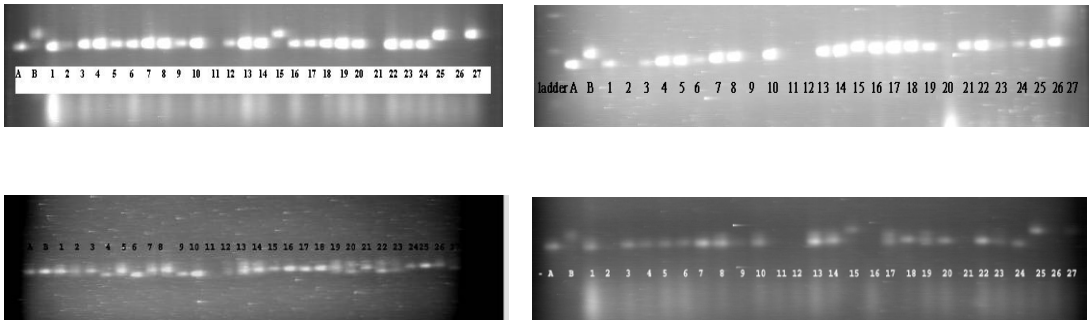


Figure 3.1: SSR polymorphism in ricebean accessions from Nepal with primers CEDG150 and CEDG118 (top), and CEDAAG002 and CEDG037 (bottom) from left to right. Lane numbers match the accession numbers given in Table 2.1. "A" refers to the ricebean, bold check, and B to the adzuki bean check.

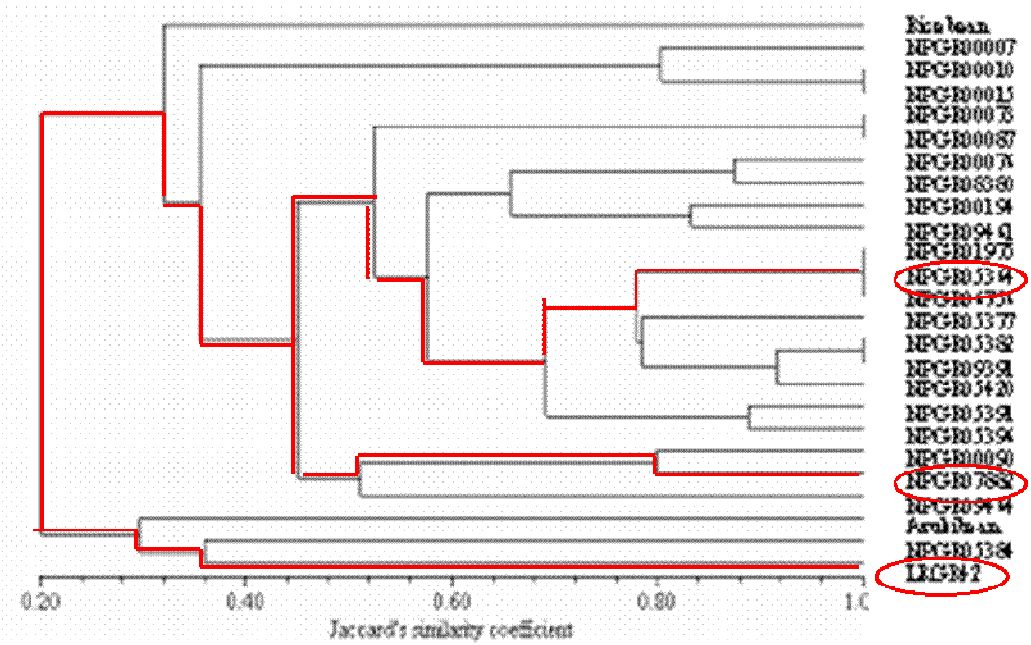
Dendrograms based on UPGMA analysis (unweighted pair group method with arithmetic average) were created using different combinations of the DNA fragments amplified generated by the polymorphic SSR primers to compare the groupings and then to identify the primers best describing the diversity in ricebean for the study. Two dendrograms are shown, based on the analysis of 13 polymorphic SSRs with PIC values greater than average PIC (0.24) (primers with bold fonts), and 8 polymorphic primers with more than 3 bands per primer (Figure 3). These primers were CEDAAG002, CEDG015, CEDG037, CEDG044, CEDG050, CEDG073, CEDG090, CEDG134, CEDG178, CEDG195, CEDG253, CEDG286 and CEDG294 (Annex 1).

The cluster analysis grouped the 23 landrace accessions and two checks of ricebean and adzuki bean into 2-4 clusters. Accessions NPGR05384, NPGR07882 and LRGR42 are from the mid to high hills in far western region of Nepal and have determinate to indeterminate

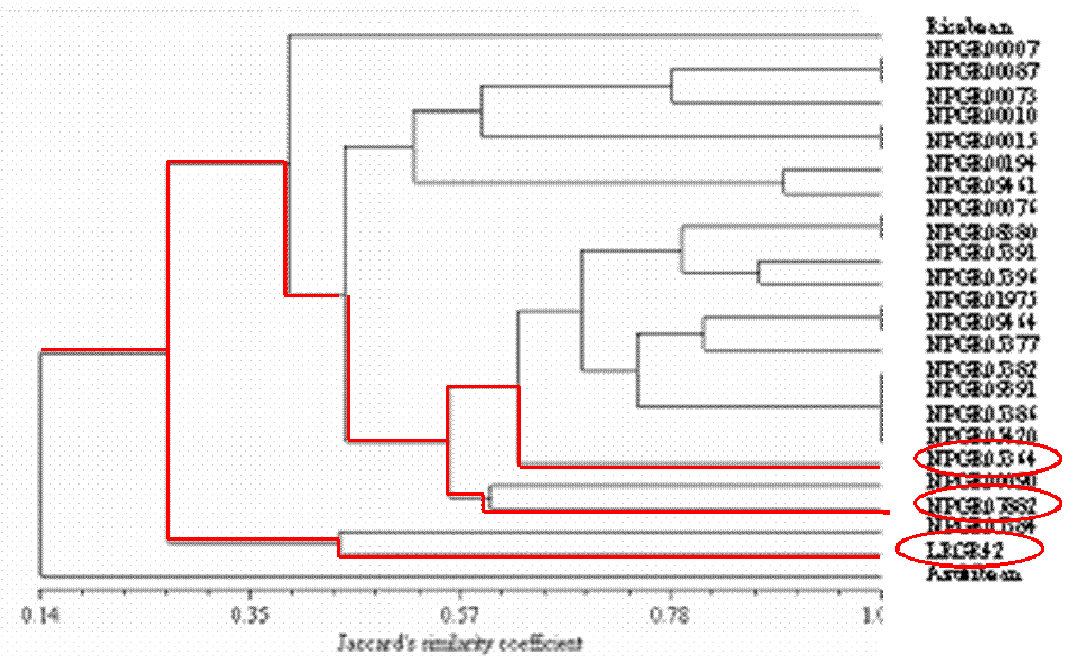
growth habit: they all clustered close to adzuki bean in dendrogram 2 (Figure 3.2 b), and two of them were close in dendrogram 1 (Figure 3.2 a). Both dendrograms were found to explain between 0.57 and 0.78 of the similarity coefficient. The second dendrogram (Figure 3.2 b) using only the 8 SSRs was found to be better at separating adzuki bean from the ricebean samples. However, we were unable to explain the ecological and agro-morphological diversity either observed during the field study or based on the passport data obtained during collection of the germplasm.

Figure 3.2: Dendrograms generated using UPGMA, showing diversity among ricebean accessions generated (a) by 13 selective polymorphic SSR primers with above average PIC values (b) by 8 polymorphic SSR primers with > poly 3 bands. Genotypes marked in red are the three from the mid to high hills noted in the text.

(a)



(b)



4. Discussion

The SSR technique has been used to quantify genetic relationships in genus *Vigna* (Wang *et al,* 2004, Han *et al,* 2005) and in several other crops. It is an efficient assay and has been found to detect more polymorphic DNA markers than RAPD. SSR are an abundant resource in the genome and have a high level of allelic diversity. They are frequently used as genetic markers in plant studies, have provided detailed information on genetic structures and gene flow in plant population studies, and are codominant in nature (Powell *et al,* 1996; McCouch *et al,* 1997; Bonin *et al,* 2001). Therefore, SSR markers were employed in the present study to detect the diversity in Nepalese ricebean landrace populations. The DNA fragments amplification by the use of adzuki bean SSR primers indicated the availability of SSR markers in ricebean that could be used in describing the genetic structure and relationships in Nepalese ricebean landraces.

Vigna umbellata is a minor cultigen limited to the marginal lands of poor farmers. Its cultivation is gradually disappearing though it has a prolific vegetative growth and high seed production and has a multiple values and there has not been carried any research activity. This is the first attempt to carry out the systematic studies on the indigenous knowledge and molecular diversity structure of this crop. Field observations of ricebean landraces in different agro-environments of Nepal showed phenotypic variations in growth habit, flowering behaviour, flower and seed size and colour and pod maturity time. These phenotypic variations were associated with a combination of agro-morphological characteristics. For example, landraces with indeterminate growth habit were found to possess prominent inflorescences with bright yellow large flowers and heavy leafy canopy. Similarly, accessions with determinate annual growth habit possessed smaller inconspicuous yellow flowers, which were phenotypically close to adzuki bean or species in *Angulares* (Seehalak *et al,* 2006).

The selected polymorphic adzuki bean SSR markers have enabled the detection of polymorphism in growth habit, though they are able to explain only a lower amount of diversity in this set of accessions. Among the 109 primers pairs with different combinations of di- and tri-nucleotide repeats (Annex 1) evaluated, only 13 primers with AG and AAG nucleotide repeats were found to be polymorphic and could be used to detect diversity in the collected Nepalese ricebean landrace populations. These primers were CEDAAG002, CEDG015, CEDG037, CEDG044, CEDG050, CEDG073, CEDG134, CEDG178, CEDG195, CEDG232, CEDG253, CEDG286 and CEDG 294. The second set of 8 primers used to produce Figure 3.2 b were useful to separate adzuki bean from ricebean, but for within-ricebean analysis the 13 SSRs with higher than average PIC were equally informative.

It is hoped to explain a high level of landrace diversity in ricebean by testing a larger number of accessions that were observed to have wide variation in the field trials in different agro-ecosystems in the 2006/07 season.

5. Conclusion

The present screening of SSR primers indicated a set of 35 SSR markers that gave good amplification of DNA in ricebean accessions. We used the adzuki bean SSR primers to attempt to detect genetic structure and relationships among the landrace accessions and provide the genetic information for breeding improved variety in ricebean. Among the primers evaluated, only 13 were found polymorphic, with an average PIC of 0.24, between the ricebean accessions and this explained some of the diversity in the ricebean accessions. These primers were distributed across the ricebean genome and they constituted di-nucleotides of AG. These primers will be used in the next activity of the project, which is to assess molecular marker diversity in ricebean landraces selected from the agro-morphological

study carried out in 2006/07 in WP3. It will also be important to assess how the grouping from this study relates to observed traits in the germplasm.

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Annex 1: List of adzuki bean SSR primers pairs screened for polymorphic *loci* (*Han et al*, 2005), showing primer sequences, primer initials, SSR motifs, approximate PCR product sizes and linkage groups.

Code	Forward Primer (5'-3')	Reverse Primer (5'-3')	Motif	Approx. bp		Linkage
				a	b	
CEDAAG002	GCAGCAACGCACAGTTTCATGG	GCAAAACTTTTCACCGGTACGACC	(AAG)16	158	179	2
CEDC009	CAGCTATACATATTGTAAAC	GTTGTCAATGTACCAGTTTG	(AC)12(AT)16	143	113	2
CEDC012	TTTAAGCAGAGACAGTTGAC	CGCCATTGTTGATATTAAGC	(AC)9(AT)17	259	234	2
CEDC014	TCCATTTCCGTGTCCATCTG	TGTTATGAAGCGCCAACG	(AC)13(AT)17	176	116	11
CEDG001	ACTATGCAGAAAGACGCTCC	GGCTCTCTCTTTCTCCATTC	(AG)26	142	122	1
CEDG002	AACTGGACCTGTACCACTGG	TACAGCCTTCTTGCACCATG	(AG)16	148	154	11
CEDG003	CCACTTTCTCTTGACTTTGC	GACCAAAGTGAAGCCAAGAG	(AG)9...(AG)13	228	222	1
CEDG005	CCAGTACCCCATATTCTTCC	CTGTGTTTGGGTTGTGATGG	(AG)22	141	145	4
CEDG007						
CEDG008	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC	(AG)26	119	101	5
CEDG011	CCCAACCAAAGCGTTTTG	CTTCTAGACTCTGAGCACTG	(AG)16 AA(AG)6	148	158	4
CEDG014	GCTTGCATCACCCATGATTC	AAGTGATACGGTCTGGTTCC	(AT)12(AG)14	137	169	5
CEDG015	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC	(AG)27	213	175	6
CEDG016	TTAGTTCACTCCGCTTGGTC	CACGTCATCCTCTGTTAGAC	(AG)26	176	154	8
CEDG018	AGCGTGTTTGTGGTGATAGC	ACACAGGAACGAACAAACCC	(AG)32	184	134	5
CEDG021	GCAGAATTTTAGCCACCGAG	AAAGGATGCGAGAGTGTAGC	(AG)26	165	159	10
CEDG022	AGGAATGTGAGATTTG	AATCGCTTCAAGGTCAAGCC	(AG)27	189	145	9
CEDG023	GCTCTCCATGAATGGAGTTG	TCATTCATTCACCCCTCC	(AG)16	81	79	5
CEDG024	CATCTTCCTCACCTGCATTC	TTTGGTGAAGATGACAGCCC	(AG)18	140	134	9
CEDG026	TCAGCAATCACTCATGTGGG	TGGGACAAACCTCATGGTTG	(AG)26	162	176	2
CEDG029	GATTGCTTTTAGCAGAGGGC	GAAGAAACCCATCTCGATCC	(AG)8	161	117	2
CEDG033						
CEDG037	GAAGAAGAACCCTACCACAG	CACCAAAAACGTTCCCTCAG	(AG)16 AC(AG)8	155	135	6
CEDG041	GCTGCATCTCTATTCTCTGG	GCCAACTAGCCTAATCAG	(AG)21	117	107	7
CEDG042	CACAGTGGTTTGGGCAACAG	TCAGAGGTTCCCATTTCCCG	(AG)15	122	162	11
CEDG043	AGGATTGTGGTTGGTGCATG	ACTATTTCCAACCTGCTGGG	(AG)14	159	191	3
CEDG044	TCAGCAACCTTGCATTGCAG	TTTCCCGTCACTCTTCTAGG	(GT)10 AT(AG)18	135	138	11
CEDG050	GGCAGAATCGTACAAGTG	GTCAGATTCTCGCTTGCATG	(AG)12	131	169	2
CEDG051	AAACATACCCCTGGCAGTTCC	TTCTGACCTAAGAAAGAGCCTGG	(AG)12	235	239	1
CEDG071	GGTCCATTGAGACGGATCGAG	TCCCACCTCAGCGGAATCC	(AG)9	249	273	8
CEDG073	CCCCGAAATTCCCCTACAC	AACACCCGCCTCTTTCTCC	(AG)24	169	175	8
CEDG077	ATCCCGTGACCCTTCTTCCT	GCTCAAGCGAAAACCCAGCA	(AG)8	180	178	4
CEDG081	TGTGGGTGTTTTATGCTTTGTG	GTATTCGGTCATTCGATCTTAC	(AG)26 AA(AG)14	191	135	10
CEDG082	CACTCAAATAGGATTTGGTTGC	ACAATGTTGCATATCCCTTTCC	(AG)18	146	136	8
CEDG084	ATCAACTGAGGAGCATCATCGA	CAACATTTCAACCTTGGGACAG	(AG)13	168	182	3
CEDG087	CCTCTTGAAATTCTCCTTGA	CCTCTTGTGAACCTCAATAA	(AG)10	129	123	1
CEDG090	ATAAGTAGAAATTGGTTCAAATG	GGTTCGTTAAAGTAACTTTTAAT	(AG)28	157	130	1
CEDG092	TCTTTTGGTTGTAGCAGGATGAAC	TACAAGTGATATGCAACGGTTAGG	(AG)17	156	192	8
CEDG098	AAAGGAGTAGAAGGTGCATA	ACAAAATTGGTTGACTCACC	(AG)5...(AG)9	112	128	11
CEDG100	CCCATCAAGTAACTACATAACA	ATGTGGGACTGGACAAATAAAA	(AG)4 A(AG)2 A(AG)3	181	115	11
CEDG102	GCCAAGGTGAACGGTGGTG	GAGCGAGAATGGCGGAAGG	(AG)29	172	146	1
CEDG103	CACCGCTGTCCATTGAAGTATTA	TCTTAGAGTGCCCTGTGAGATTG	(AG)37	126	98	4
CEDG104	TATGGCCCGAGCAAACCTTG	CCGTTCGGTCTTCGGTTGAA	(AG)13	143	141	11
CEDG111	TGGAAGTTTCCAAGAGGGTTTTC	TCTCACCACCTTTTACCTTCTCA	(AT)7(AG)14	202	216	7
CEDG112	GCAATATTCGCATTATTCATTCA	GTGTTTCAAAGCACTATACTTAA	(AT)18(AG)20	164	168	8
CEDG114	GAACCTTGATGAAGGGGTAA	GATCACAAAGCAAAGCACA	(AG)20(AT)8(	231	361	5

		T	GT)8			
CEDG116	TTGTATCGAAACGACGACGCA GAT	AACATCAACTCCAGTCTCAC CAAA	(AG)4 AA(AG)12	160	160	ND
CEDG117	GTACACTTCCACTAATCCAAAA TT	TGGTACCTTCCTTATCTGAA ATTA	(AG)21(AT)30	168	129	3
CEDG118	AACCCAACCAACCCTTGTGGTA AG	GCTGGAATCATAATACCGCC TTGT	(AG)21	190	130	6
CEDG121	CTTTCAAAATAATGTTGAGGCA TA	CAATACATAAATAACCTTTT CTGC	(AG)18	80	86	6
CEDG124	AGCAAATTATTGGATGAAAG	TTATTTGGAATACGGATTGT	(AG)9	223	221	5
CEDG125	TGGAATATACTGTTTAATAGAG	AGATTAATTTGATCACTCAT TC	(AT)14(AG)12	202	226	8
CEDG127	GGTTAGCATCTGAGCTTCTTCG TC	CTCCTCACTTGGTCTGAAAC TC	(TG)3(AG)9	258	280	4
CEDG131	CCTTTTTCTTCCTACCCTCTACC	CACCACCTAGCTGTTGCTAG	(AG)12	153	171	7
CEDG134	CTCCGTGTTGAAACAATGACG	GGTCTTTCTGATCTACGAAC TTG	(AG)11	217	201	10
CEDG138	CATTCTGATGAAAAGATCAAG G	CAATGTAACAGACTCACTGG	(AG)21	259	205	1
CEDG141	CCAGGCATCCATGATGACC	GAAGTTGTTGGTAATGGTTG CCTC	(AT)6(AG)13	167	179	1
CEDG146	GGTGATCGGATTTCAGAG	GGAGAAGAGAATAGAGACG	(AG)9	122	118	6
CEDG147	CTCCGTCGAAGAATTGGTTGAC	GCAAAAATGTGGCGTTTGGT TGC	(AG)10	276	330	10
CEDG150	GAAGGGAATGAAAATGAAACC C	GTTCAATCCATTCAGTCTCC	(AG)14	182	194	10
CEDG151	GTAGAACAGTTATGACACATG	TGTTAACTTCGTTGGGTACA C	(AC)6(AT)4(A GAT)3(AG)17	180	166	8
CEDG154	GTCCTTGTTTTCCTCTCCATGG	CATCAGCTGTTCAACACCCT GTG	(AG)14	212	232	4
CEDG158	GGTCAACAGGAGAGTTAG	CCACCTCCTCATTACCATTC	(AG)19	132	116	5
CEDG165	GCTCTGTCAGTTCCCACTAC	GGTCCTGAACCCAGATGAAC	(AG)10	146	142	4
CEDG166	GGTACAACATTCTTCTATTTG	GGCTTATGAGTTTATCTTATC	(AT)12(AG)18	228	208	9
CEDG173	GATAAGAGATGCATCACTC	CTTCTCTTCCATCACATCTG	(AG)23	124	110	9
CEDG174	GAGGGATCTCCAAAGTTCAAC GG	GAAGGCTCCGAAGTTGAAG GTTG	(AG)22	215	191	7
CEDG178	CGGAAGAAGAACGCAGAGTG	GCATCAACAAGGACTTCTGC	(AG)10 G(AG)5	139	137	1
CEDG180	GGTATGGAGCAAAACAATC	GTGCGTGAAGTTGTCTTATC	(AG)11	126	122	10
CEDG183	CTCATGGTGCTACCAACCTTGA C	CCATCGCCAACGAAGTTGGT C	(AG)17	166	156	11
CEDG186	GGATGGGAGAGTAAGAAG	GCATGGCATGATGACTTG	(AG)18	180	156	3
CEDG191	CAATAAGCAATCTGTGGAGAG	CTGCAGGAAACTTGGAATTG C	(AG)21	155	149	6
CEDG195	GAGGGTCTCCACTTTTGAAACC C	GATACTAAGGCTTTCTCCAC CCAC	(AG)11	141	127	6
CEDG201	CGGGTAGACAAAGAGATACAC G	CTAGCAGAAACAGGAGATC CTC	(AG)10	161	167	7
CEDG202	GTTGGAGTCTTGCACTGCG	CTATCCCCTGATCAGGAGC	(AG)12	166	182	8
CEDG203	GACTGAACCTATGCGGTCCAAC	CAACGTGTTAGCCTTCTTGC CTC	(AG)11	136	126	7
CEDG204	CCTTGGTTGGAGCAGCAGC	CACAGACACCCTCGCGATG	(AG)15	156	164	1
CEDG205	GTGGTGGTGACAGTAGCAGTA G	CAGCCACCACAAGACAACCT C	(AG)4 AT(AG)11	151	171	3
CEDG210	GAACCCACTTCTGAAGTTC	GAACAACCTCTGCAGTAG	(AG)10	188	212	2
CEDG212	CTTAAGGCAGATTACCTG	GCAACGCAAGTTATCAAG	(AG)22	250	228	5
CEDG214	CACTCACTGCAAAGAGCAAC	CTACCTATCTGAGGGACAC	(AG)4 AA(AG)31	193	185	1
CEDG225	GAGGAAGTGTTGCAGCACC	GTAGACTCTGCAGAGGGATG	(AG)8 TG(AG)3(TG)2 (AG)4	143	163	2
CEDG228	GTCGTTTCCGGAAACTGTTC	GATCCGAACCTCTTTCTGC	(AG)17	197	219	9
CEDG232	GATGACCAAGGTAACGTG	GGACAGATCCAAAACGTG	(AG)16	149	173	4
CEDG238	GCAGAATTTGACTGCTAGAAA GC	CCATACATTTGTTGCACGCA TG	(AG)12	161	173	9
CEDG241	GTGACCCACTAAATTTCGTG	GAACTGGCTATTCCGGTAAC	(AG)5	260	266	1
CEDG245	GATAGAGCTTAAACCCTC	CTTTTGATGACAAATGCCC	(AC)10(AT)9(A G)14	146	196	6
CEDG247	GTAGACACTGATCATCACC	GACCATCATCGATACGATTC	(AG)16	149	169	8
CEDG248	CAGAACACAAAAGGGTTCTCG	GTGGATTCACTCGCTTCC	(AG)17	108	130	6
CEDG251	ATATCTCAAAACCCTTCCTG	CCTCAATAACAATGATACGA C	(AG)12	190	222	8
CEDG253	CACTTCCATGATGACTCACC	CACCCTTCTTTATCCTCTTCG	(AG)30	236	216	5

CEDG254	CGATGTCTCTTGCTTCAAGG	GTGAAGGACTAGCCAAGTTTG	(AT)13(AG)11	142	174	1
CEDG257	GACTACTCTCAAGACCAAAG	GATGGTTGTAGATAACACTCC	(AG)12	109	107	8
CEDG259	GATCATCGGACAGAGCTTCC	CACTCTCTGCGAACTCAATCG	(AG)11	142	138	9
CEDG263	GATTGGGAATCTGCTGTTG	GTGATCCACACACAGTAC	(AG)6 AT(AG)7	138	122	1
CEDG264	GATTCCCTTCCTAGCTATGG	CTGCTGGACATGAAGATTCAG	(AG)10 AT(AG)16	197	203	5
CEDG268	CATCTCCCTGAAACTTGTG	GCTATCAATCGAGTGCAG	(AG)16	171	145	5
CEDG269	CTGTTACGGCACCTGGAAAG	GCAGAGACACACCTTAACCTTG	(AG)14	190	184	8
CEDG279	GGTCTTTCTAAGCGGAGCAC	CTGCCTCTCTACACAAGTGG	(AG)5(AAAG)2(AG)3G(AG)9	184	182	11
CEDG280	CAGATTCAGTCTGCTTTTGAG	CCACTGCATTCATTCATGAG	(AG)14	175	203	10
CEDG282	CAGCAACAAGACATGGAGTG	GGTGACCACTTAGACAGAC	(AT)16(AC)5(AG)10	133	153	6
CEDG286	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC	(AG)23	227	205	8
CEDG287	CCTTATACTAAAGATGTTGGTGG	GTGATACGCATATAGGTTCAC	(AG)14	158	148	11
CEDG290	GACACTCTTTGTTTGTAGG	CAGTGATCACTCTGGTTG	(AG)11	139	133	7
CEDG292	GTGGTTTTGTTGACCTTGTC	GTAATGCTCCAATGGCTTC	(AG)6	160	146	4
CEDG294	CACCTTCTTAATCTCTTCACC	GGGTTTCTCTTAATTCATTGAGTC	(AT)27(AG)15	213	231	3
CEDG304	ACCACTTCATAATCCCTGAG	GTTGCATGCTATATTTTGGTTCAC	(AG)9	82	84	9
CEDG305	GCAGCTTCACATGCATAGTAC	GAACTTAACTTGGGTTGTCTGC	(AG)22	124	134	3
CEDGAG001	CTCATCAGGGACATCCTCCC	GATCGTGATCGATCCAACGGTC	(GAG)4	172	166	9

a = product size of *V. angularis*; b = product size of *V. nepalensis*; ND = not determined

Annex 2: List of ricebean landrace accessions collected from different parts and growing environments in Nepal

S No	Accession number	Collection districts	Local names	Collection institute
1	NPGR-00007	Nuwakot	Chhirbire masyang	ABD
2	NPGR-00008	Nuwakot	Panhelo masyang	ABD
3	NPGR-00010	Lalitpur	Masyang	ABD
4	NPGR-00012	Nuwakot	Rato masyang	ABD
5	NPGR-00015	Bhaktapur	Masyang	ABD
6	NPGR-00073	Gulmi	Thulo panhelo masyang	ABD
7	NPGR-00076	Arghakhanchi	Dhawanse masyang	ABD
8	NPGR-00087	Pyuthan	Masyang	ABD
9	NPGR-00090	Dang	Jhilinge masyang	ABD
10	NPGR-00194	Kabre	Masyang	ABD
11	NPGR-01975	Baitadi	Baramase masyang	ABD
12	NPGR-05364	Bhojpur	Masyang	ABD
13	NPGR-05368	Bhojpur	Bhage masyang	ABD
14	NPGR-05370	Terhathum	Rato ghore	ABD
15	NPGR-05373	Gorkha	Masyang	ABD
16	NPGR-05377	Lamjung	Gurans	ABD
17	NPGR-05382	Tanahu	Masyang	ABD
18	NPGR-05384	Mugu	Gurans	ABD
19	NPGR-05386	Humla	Gurans	ABD
20	NPGR-05391	Bajura	Ghore mas	ABD
21	NPGR-05396	Illam	Banmara masyang	ABD
22	NPGR-05420	Dhankuta	Ghore mas	ABD
23	NPGR-05423	Dhankuta	Seto mas	ABD
24	NPGR-05432	Baitadi	Gurans	ABD
25	NPGR-05565	Okhaldhunga	Masyang	ABD
26	NPGR-06591	Mugu	Masyang	ABD
27	NPGR-06657	Kalikot	Rato masysng	ABD
28	NPGR-06756	Humla	Gurans	ABD
29	NPGR-07583	Jhapa	Masyang	ABD
30	NPGR-07882	Bajhang	Masyang	ABD
31	NPGR-08380	Myagdi	Syaltung	ABD
32	NPGR-08382	Banglung	Syaltung	ABD
33	NPGR-09391	Syangja	Masyang	ABD
34	NPGR-09461	Panchthar	Masyang	ABD
35	NPGR-09464	Taplejung	Masyang	ABD
36	LRGR42	Surkhet	Siltung	LIBIRD
37	LRGR43	Surkhet	Siltung	LIBIRD
38	LRGR44	Surkhet	Siltung	LIBIRD
39	LRGR75	Pyuthan	Raiyans	LIBIRD
40	LRGR91	Dang	Siltung	LIBIRD
41	LRGR99	Palpa	Jhilinge	LIBIRD
42	LRGR101	Palpa	Jhilinge	LIBIRD
43	LRGR102	Palpa	Jhilinge	LIBIRD
44	LRGR103	Palpa	Jhilinge	LIBIRD
45	LRGR107	Palpa	Jhilinge	LIBIRD
46	LRGR111	Gulmi	Jhilinge	LIBIRD
47	LRGR117	Gulmi	Jhilinge	LIBIRD
48	LRGR129	Palpa	Siltung	LIBIRD
49	LRGR137	Kaski	Masyang	LIBIRD
50	LRGR152	Kavre	Masyang	LIBIRD
51	NPGR-00006	Nuwakot	Masyang	ABD
52	NPGR-00009	Nuwakot	Pahelo masyang	ABD
53	NPGR-00011	Nuwakot	Masyang	ABD
54	NPGR-00013	Nuwakot	Dhade kalo masyang	ABD
55	NPGR-00074	Arghakhanchi	Khaire masyang	ABD
56	NPGR-00092	Dang	Jhilinge seto masyang	ABD
57	NPGR-00093	Pyuthan	Chhirbire masyang	ABD
58	NPGR-00193	Kabhre	Masyang	ABD
59	NPGR-00195	Kabhre	Masyang	ABD
60	NPGR-00197	Kabhre	Masyang	ABD
61	NPGR-00198	Kabhre	Masyang	ABD
62	NPGR-00199	Kabhre	Masyang	ABD
63	NPGR-01972	Ilam	Masyang	ABD
64	NPGR-01974	Ilam	Seto masyang	ABD
65	NPGR-05367	Bhojpur	Rato masyang	ABD

66	NPGR-05371	Bhojpur	Naga masyang	ABD
67	NPGR-05376	Gorkha	Masyang	ABD
68	NPGR-05380	Lamjung	Masyang	ABD
69	NPGR-05381	Lamjung	Thulo masyang	ABD
70	NPGR-05383	Tanahun	Masyang	ABD
71	NPGR-05394	Dhankuta	Rato masyang	ABD
72	NPGR-05397		Masyang	ABD
73	NPGR-05398	Ilam	Masyang	ABD
74	NPGR-05399	Ilam	Masyang	ABD
75	NPGR-05400	Ilam	Masyang	ABD
76	NPGR-05401	Ilam	Masyang	ABD
77	NPGR-05407	Ilam	Masyang	ABD
78	NPGR-05408	Ilam	Masyang	ABD
79	NPGR-05409	Ilam	Masyang	ABD
80	NPGR-05411	Ilam	Masyang	ABD
81	NPGR-05412	Dhankuta	Masyang	ABD
82	NPGR-05415	Dhankuta	Masyang	ABD
83	NPGR-05416	Dhankuta	Masyang	ABD
84	NPGR-05417	Dhankuta	Ghore mas	ABD
85	NPGR-05425	Dhankuta	Masyam	ABD
86	NPGR-05429	Ilam	Masyam	ABD
87	NPGR-05430	Baitadi	Masyang	ABD
88	NPGR-05435		Gurans	ABD
89	NPGR-06725	Humla	Gurans	ABD
90	NPGR-07883	Bajura	Masyang	ABD
91	4	Dailekh	Gurans (Masyang)	ABD
92	5	Ilam	Thulo Masyang	ABD
93	6	Kathamndu	Masyang	ABD
94	NPGR-08381	Myagdi	Syaltung	ABD
95	NPGR-08383	Baglung	Syaltung	ABD
96	NPGR-09462	Panchthar	Masyang	ABD
97	NPGR-09466	Taplejung	Rato masyang	ABD
98	NPGR-09691	Bajura	Gurans	ABD
99	NPGR-09710	Bajhang	Gurans	ABD
100	NPGR-10476	Gulmi	Jhilinge	ABD
101	NPGR-00004	Nuwakot	Masyang	ABD
102	NPGR-00005	Nuwakot	Masyang	ABD
103	NPGR-00014	Nuwakot	Dhade rato masyang	ABD
104	NPGR-00072	Gulmi	Pahenlo rato masyang	ABD
105	NPGR-00075	Arghakhanchi	Dhani masyang	ABD
106	NPGR-00088	Pyuthan	Chhirbire masyang	ABD
107	NPGR-00089	Dang	Khaire masyang	ABD
108	NPGR-00091	Dang	Chhirbire masyang	ABD
109	NPGR-00183	Kabhre	Masyang	ABD
110	NPGR-00184	Kabhre	Masyang	ABD
111	NPGR-00185	Kabhre	Masyang	ABD
112	NPGR-00186	Kabhre	Masyang	ABD
113	NPGR-00187	Kabhre	Masyang	ABD
114	NPGR-00188	Kabhre	Masyang	ABD
115	NPGR-00189	Kabhre	Masyang	ABD
116	NPGR-00190	Kabhre	Masyang	ABD
117	NPGR-00191	Kabhre	Masyang	ABD
118	NPGR-00192	Kabhre	Masyang	ABD
119	NPGR-01973	Ilam	Banmara masyang	ABD
120	NPGR-05365	Bhojpur	Seto masyang	ABD
121	NPGR-05366	Bhojpur	Kalo masyang	ABD
122	NPGR-05372	Gorkha	Masyang	ABD
123	NPGR-05374	Gorkha	Masyang	ABD
124	NPGR-05378	Lamjung	Gurans	ABD
125	NPGR-05379	Lamjung	Masyang	ABD
126	NPGR-05388	Humla	Gurans	ABD
127	NPGR-05392	Bajura	Rangale mas	ABD
128	NPGR-05393	Dhankuta	Seto masyang	ABD
129	NPGR-05395		Thulo rato ghore	ABD
130	NPGR-05402	Ilam	Masyang	ABD
131	NPGR-05403	Ilam	Masyang	ABD
132	NPGR-05404	Ilam	Masyang	ABD
133	NPGR-05405	Ilam	Masyang	ABD
134	NPGR-05406	Ilam	Masyang	ABD
135	NPGR-05413	Dhankuta	Masyang	ABD
136	NPGR-05414	Dhankuta	Masyang	ABD

137	NPGR-05418	Dhankuta	Ghore mas	ABD
138	NPGR-05419	Dhankuta	Ghore	ABD
139	NPGR-05422	Dhankuta	Masyam	ABD
140	NPGR-05424	Dhankuta	Masyam	ABD
141	NPGR-05428		Gurans	ABD
142	NPGR-05431			ABD
143	NPGR-05436	Baitadi	Gurans	ABD
144	1	Ramechhap	Masyang	ABD
145	2	Dailekh	Situng	ABD
146	3	Kavre Palanchok	Masyang	ABD
147	NPGR-09463	Panchthar	Masyang	ABD
148	NPGR-09465	Taplejung	Masyang	ABD
149	NPGR-09690	Bajura	Gurans	ABD
150	NPGR-10459	Arghakhanchi	Jhilange	ABD
151	LRGR 3	Dadeldhura	Gurans	LI-BIRD
152	LRGR 4	Dadeldhura	Gurans	LI-BIRD
153	LRGR 5	Dadeldhura	Gurans	LI-BIRD
154	LRGR 7	Dadeldhura	Gurans	LI-BIRD
155	LRGR 8	Dadeldhura	Gurans	LI-BIRD
156	LRGR 9	Dadeldhura	Gurans	LI-BIRD
157	LRGR 10	Dadeldhura	Gurans	LI-BIRD
158	LRGR 14	Doti	Gurans	LI-BIRD
159	LRGR 18	Doti	Gurans	LI-BIRD
160	LRGR 24	Bajura	Gurans	LI-BIRD
161	LRGR 25	Bajura	Gurans	LI-BIRD
162	LRGR 30	Bajura	Gurans	LI-BIRD
163	LRGR 35	Achham	Gurans	LI-BIRD
164	LRGR 37	Achham	Gurans	LI-BIRD
165	LRGR 47	Darchula	Gurans	LI-BIRD
166	LRGR 54	Baitadi	Gurans	LI-BIRD
167	LRGR 55	Baitadi	Gurans	LI-BIRD
168	LRGR 72	Pyuthan	Jhilunge	LI-BIRD
169	LRGR 73	Pyuthan	Raiyans	LI-BIRD
170	LRGR 79	Dang	Siltung	LI-BIRD
171	LRGR 82	Dang	Siltung	LI-BIRD
172	LRGR 83	Dang	Jhilunge	LI-BIRD
173	LRGR 84	Dang	Siltung	LI-BIRD
174	LRGR 87	Dang	Siltung	LI-BIRD
175	LRGR 88	Dang	Siltung	LI-BIRD
176	LRGR 89	Dang	Siltung	LI-BIRD
177	LRGR 90	Dang	Siltung	LI-BIRD
178	LRGR 93	Nuwakot	Gurans	LI-BIRD
179	LRGR 94	Nuwakot	Kalo masyang	LI-BIRD
180	LRGR 95	Nuwakot	Khairo masyang	LI-BIRD
181	LRGR 97	Nuwakot	Masyang	LI-BIRD
182	LRGR 100	Palpa	Jhilinge	LI-BIRD
183	LRGR 108	Gulmi	Jhilinge	LI-BIRD
184	LRGR 113	Gulmi	Jhilinge	LI-BIRD
185	LRGR 116	Gulmi	Jhilinge	LI-BIRD
186	LRGR 118	Gulmi	Jhilinge	LI-BIRD
187	LRGR 120	Palpa	Jhilinge	LI-BIRD
188	LRGR 123	Palpa	Jhilinge	LI-BIRD
189	LRGR 124	Palpa	Jhilinge	LI-BIRD
190	LRGR 126	Palpa	Jhilinge	LI-BIRD
191	LRGR 128	Palpa	Jhilinge	LI-BIRD
192	LRGR 130	Palpa	Siltung	LI-BIRD
193	LRGR 131	Palpa	Siltung	LI-BIRD
194	LRGR 132	Gulmi	Jhilinge	LI-BIRD
195	LRGR 133	Palpa	Jhilinge	LI-BIRD
196	LRGR 134	Palpa	Jhilinge	LI-BIRD
197	LRGR 135	Palpa	Jhilinge	LI-BIRD
198	LRGR 136	Kaski	Masyang	LI-BIRD
199	LRGR 138	Kaski	Masyang	LI-BIRD
200	LRGR 139	Kaski	Masyang	LI-BIRD
201	LRGR 141	Kaski	Masyang	LI-BIRD
202	LRGR 142	Kaski	Masyang	LI-BIRD
203	LRGR 143	Kaski	Masyang	LI-BIRD
204	LRGR 144	Kaski	Masyang	LI-BIRD
205	LRGR 145	Kaski	Masyang	LI-BIRD
206	LRGR 146	Kaski	Masyang	LI-BIRD
207	LRGR 148	Kavre	Masyang	LI-BIRD

208	LRGR 151	Kavre	Masyang	LI-BIRD
209	LRGR 153	Kavre	Masyang	LI-BIRD
210	LRGR 154	Nuwakot	Masyang	LI-BIRD
211	LRGR 155	Nuwakot	Masyang	LI-BIRD
212	LRGR 156	Nuwakot	Masyang	LI-BIRD
213	LRGR 157	Kaski	Masyang	LI-BIRD
214	LRGR 158	Kaski	Masyang	LI-BIRD
215	LRGR 159	Kaski	Masyang	LI-BIRD
216	LRGR 160	Kaski	Masyang	LI-BIRD