Annals of the Sri Lanka Department of Agriculture. 2004. 6:177-186.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF LOCALLY COLLECTED WILD RICE GERMPLASM

P.K. SAMARAJEEWA, R.M.R. NILANTHI
and
A.S.U. LIYANAGE
Plant Genetic Resources Centre, Gannoruwa, Peradeniya

ABSTRACT

Wild rice species are considered to be a rich source of genes for pest and disease resistance as well as some abiotic stresses such as tolerance for certain adverse soil conditions. It is important to collect and conserve the maximum genetic richness of the local wild rice germplasm. For this, analysis of genetic diversity can only provide necessary data. Towards achieving these objectives, locally collected wild species of rice were characterized based on morphological markers and RAPD (Random Amplified Polymorphic DNA) markers. Eighteen wild rice accessions belonging to *Oryza eichingeri*, *O. nivara*, *O. rhizomatis* and *O. rufipogon* which had been collected from different locations of Sri Lanka and two accessions of one introduced species *O. glaberima* were used for the analysis along with cultivated variety, Bg 94-1 and traditional variety, Suduru samba both belonging to *O. sativa*. Morphological and RAPD data were used to construct dendrograms of the accessions following the computer software SPSS. Twenty-two accessions could be separated into two major groups based on genetic distance between as well as within the species revealing their genetic groups. *Oryza nivara* and *O. rufipogon* were closely related to *O. sativa* than the other species. RAPD method discriminated accessions better than morphological markers. All the accessions could be separated based on DNA data. There was a marked genetic diversity among the individuals of each species even though they were collected from the same locality. Though the collection was very small, *O. eichingeri* had accessions with the highest level of diversity.

KEY WORDS: Characterization, Germplasm, Morphology, RAPD, Wild rice.

INTRODUCTION

Wild rice, related to cultivated rice, *Oryza sativa*, has been recognized as an important constituent in any rice genetic resource conservation program. Evaluation of wild rice germplasm has shown that resistance to certain pests is widely scattered among different species of the genus. For example, stem borer resistance has been found in 13 species of wild rice and brown plant hopper resistance in 11 species (Heinrichs *et al*, 1985, Ikeda *et al*. 1990, Khan *et al*., 1991). Resistance to sheath blight and bacterial leaf blight are lacking in cultivated rice, but found in some wild species (Amante *et al*., 1990; Ikeda *et al*, 1990). Resistance to abiotic stresses such as salinity and deep-water tolerance and other useful special traits such as cytoplasmic male sterility are found in some wild species (IRRI, 1990; Vermani and Shinjvo, 1988). Apart from the above features wild rice grain has a high protein and carbohydrate content, and very low fat content. The nutritional quality of wild rice appears to equal or surpass that of other cereals (Oelke *et al*., 1997).

About 22 wild rice species have been reported from various parts of the world and they belong to different genomic groups (Gurdev, 1991) Accordingly *O. sativa*, *O. nivara*, *O. rufipogon* and *O. glaberrima* belong to genomic group AA and *O. eichingeri* and *O rhizomatis* belong to genomic group CC. In Sri Lanka, five wild rice species have been reported, *ie. O. eichingeri*, *O. granulata*, *O. nivara*, *O. rufipogon* and *O. rhizomatis* (Vaughan, 1994). They grow in diverse habitats in the country. *Oryza nivara* has been found in environmentally vastly different locations. Most of these habitats except in strictly reserved areas are under threat of destruction, posing danger to the survival of wild germplasm.

Since the inception of conservation of germplasm by the Plant Genetic Resources Center (PGRC), a number of accessions of wild rice germplasm have been collected and seeds have been stored in the Gene Bank. However, genetic diversity or genetic richness of these locally collected germplasm has not been assessed to support any future collection strategy.

The objective in this study is to analyze the genetic diversity of the wild rice germplasm collected by the PGRC using two methods, morphological and molecular markers. Molecular markers are identified to be useful tools in analyzing the genetic diversity in plant populations at the DNA level (Yoon *et al.*, 2000). Molecular markers are also helpful in understanding the phylogenetic relationships between plant species within a given taxonomic group (Fatokun *et al.*, 1993; Kaga 1996a). Even duplicate accessions can be distinguished using molecular markers (Virk *et al.*, 1995).

In the present study, we used Random Amplified Polymorhpic DNA as a molecular method together with morphological markers to characterize 22 accessions in the PGRC collection representing five wild rice species, *O. eichingeri*, *O. glaberima*, *O. nivara*, *O. rhizomatis* and *O. rufipogon* and two cultivated types.

MATERIALS AND METHODS

Plant materials

Seeds of the eighteen accessions of locally collected wild rice, two accessions of introduced rice, one accession of cultivated rice and one accession of cultivated traditional rice were obtained from the gene bank of PGRC (table 1). Each accession was given a code name from 1-22. The Exploration Division of PGRC had collected these from different parts of the country. Seeds were planted in pots in the plant house of PGRC and five plants from each accession were maintained. Two weeks after germination, immature leaves were harvested from each accession for the DNA extraction.

Table 1. Germplasm collection used in the present study.

Code	*Species*	*Place of collection*
1	*O. eichingeri*	Rathnapura
2	*O. eichingeri*	Rathnapura
3	*O. eichingeri*	Rathnapura
4	*O. glaberrima*	Introduced
5	*O. glaberrima*	Introduced
6	*O. nivara*	Kegalle
7	*O. nivara*	Anuradhapura
8	*O. nivara*	Batticaloa
9	*O. nivara*	Galle
10	*O. nivara*	Badulla
11	*O. nivara*	Matale
12	*O. nivara*	Anuradhapura
13	*O. nivara*	Anuradhapura
14	*O. rhizomatis*	Pollonnaruwa
15	*O. rhizomatis*	Puttalum
16	*O. rhizomatis*	Puttalum
17	*O. rhizomatis*	Puttalum
18	*O. rufipogon*	Colombo
19	*O. rufipogon*	Matara
20	*O. rufipogon*	Badulla
21	*O. sativa* (Bg 94-1)	-
22	*O. sativa* (Suduru samba)	-

Morphological analysis

Morphological analysis was done by using both quantitative and qualitative characters. Seedling height, leaf blade length, leaf blade width, ligule length at late vegetative stage, days to heading, culm length, culm number after full heading, culm diameter at flowering period, panicle length, 100 grain weight at maturity, grain length and grain width were measured as quantitative characters. At late vegetative stage leaf blade pubescence, leaf blade colour, basal leaf sheath colour, leaf angle, flag leaf angle, ligule color, ligule shape, collar colour, auricle colour were measured. Culum angle after flowering, internode colour after flowering, panicle type at near maturity, secondary branching, panicle exertions, seed coat colour at maturity were the other qualitative characters evaluated. Morphological data were analyzed by using software package SPSS.

DNA extraction

Total genomic DNA was extracted using CTAB method according to Doyle and Doyle (1990) with slight modifications. 120 mg of young fresh leaf tissue was ground to a fine powder using liquid nitrogen, and then 700 µl of extraction buffer was added. The extraction buffer consisted of 3% of CTAB

(cetyltrimethyl ammonium bromide), 2 M NaCl, 100 mM Tris- HCl (pH 8), 1% ß-mercaptoethanol, 20 mM EDTA and 2% PVP (polyvinyl pyrrolidone). The homogenate was incubated at 65°C for 30 minutes and centrifuged at 13,500 g for 10 minutes. The supernatant was transferred to a new tube and 700 µl of chloroform-isoamyl alcohol mixture (24:1 v/v) was added to it. The content was then mixed by gently turning the tubes upside-down and subsequently the mixture was centrifuged at 13,500 g for 10 minutes. After centrifugation the supernatant was transferred to a new tube. Chloroform-isoamyl alcohol (600 µl) was again added, and then centrifuged at 13,500 g again for 10 minutes and supernatant was transferred to a new tube. DNA was precipitated by mixing with 0.6 volume of ice-cold isopropanol, and then pelleted by centrifugation at 13,500 g for 10 minutes. The supernatant was removed, the pellet was washed with 70% ethanol to remove salts, dried in a speed-vac and dissolved in 25 µl of TE buffer [10 mM Tris-HCl(pH8) ,0.001M EDTA(pH 8)]. This was treated with RNAse (Sigma, 10 µg/ml) at 37°C for 30 minutes. The quality of the DNA was checked by 0.8% agarose gel electrophoresis in 0.5X TBE buffer [0.045 M Tris-borate, 0.001 M EDTA (pH 8.0)], stained with ethidium bromide (0.5µg/ml) and observed under UV transilluminator. The DNA samples were quantified by Shimadzu UV-1201spectrophotometer. Optical density (OD) readings were obtained at 260nm and 280nm. DNA quantity was estimated based on 260nm reading.

RAPD analysis

Selection of random primers

In order to select primers that can give rise to PCR products in wild rice, RAPD analysis was carried out using 20 tenmer primers (Operon technologies Inc. USA). Out of this, 10 primers that gave rise to amplified products were selected for evaluating genetic relationship of wild rice accessions.

PCR amplification

PCR was performed according to Williams *et al.* (1990) by using Perkin- Elmer (480) DNA thermocycler. The reaction consisted of 1µl of template DNA (25ng/µl), 1µl of dNTP mixture (2.5mM), 0.025 µl of *Taq* polymerase (Takara Schu Japan, 5 units/µl), 1µl of primer and 1.25µl of 10X PCR buffer in 12.5µl volume. The amplification condition included a total of 40 cycles with 1 min (3 min for the fist cycle) at 94^{0}C for template denaturation, three min at 35 ^{0}C for primer annealing and 2 min (10 min for the final cycle) at 72 ^{0}C for primer extension. Finally the reaction mixture was subjected to soaking at 4 ^{0}C.

Observation of amplified products

Amplified DNA samples were electrophoresed on 1.4% agarose gels in 0.5X TBE buffer at 4v/cm for 3 hours. One kb DNA ladder was used as a size marker (Takara Schuzo, Japan). The gels were stained with ethidium bromide (0.5µg/ml) and observed under UV transilluminator. Permanent records were made on instant film (Polaroid 667). In addition, gels were scanned under Bio Image Gel Print 2000 i/vga electrophoresis image analyzing system.

Data analysis

The banding patterns from RAPD analysis for 10 Operon primers that generated polymorphism were recorded by visual observation. The presence of amplified products (bands) in each position was recorded as 1 and absence as 0. The total number of bands scored for each primer tested is given in table 2. The 0/1 matrix was used to determine the confidence limits of the UPGMA based (Unweigted pair - group method with arithmetic means) dendrogram.

RESULTS AND DISCUSSION

Morphological analysis

Since we assessed a large number of morphological characters they are not presented here. The dendrogram constructed using morphological data is shown in Figure 1. The results indicate diversity among the species of wild rice. Although the accessions could be differentiated at species level, accessions within a species could not be separated.

RAPD analysis

Identification of suitable primers and scoring

Of the 20 primers used in this study, only 15 amplified wild rice DNA. From these, data generated from 10 primers that showed a high degree of polymorphism were selected, for data analysis. RAPD profile of 20 accessions with the primer OPA-20 is given in Figure. 2. Each species is characterized by the presence of a distinct band. Similarly amplification fragments given with the other nine primers were observed. The number of bands for all primers ranged from 15 to 24 with an average of 20 bands per primer (table 2). Out of the 195 bands scored 168 were polymorphic and shared between at least two individuals. Seventeen bands were polymorphic and unique to a single wild rice species while 10 were monomorphic.

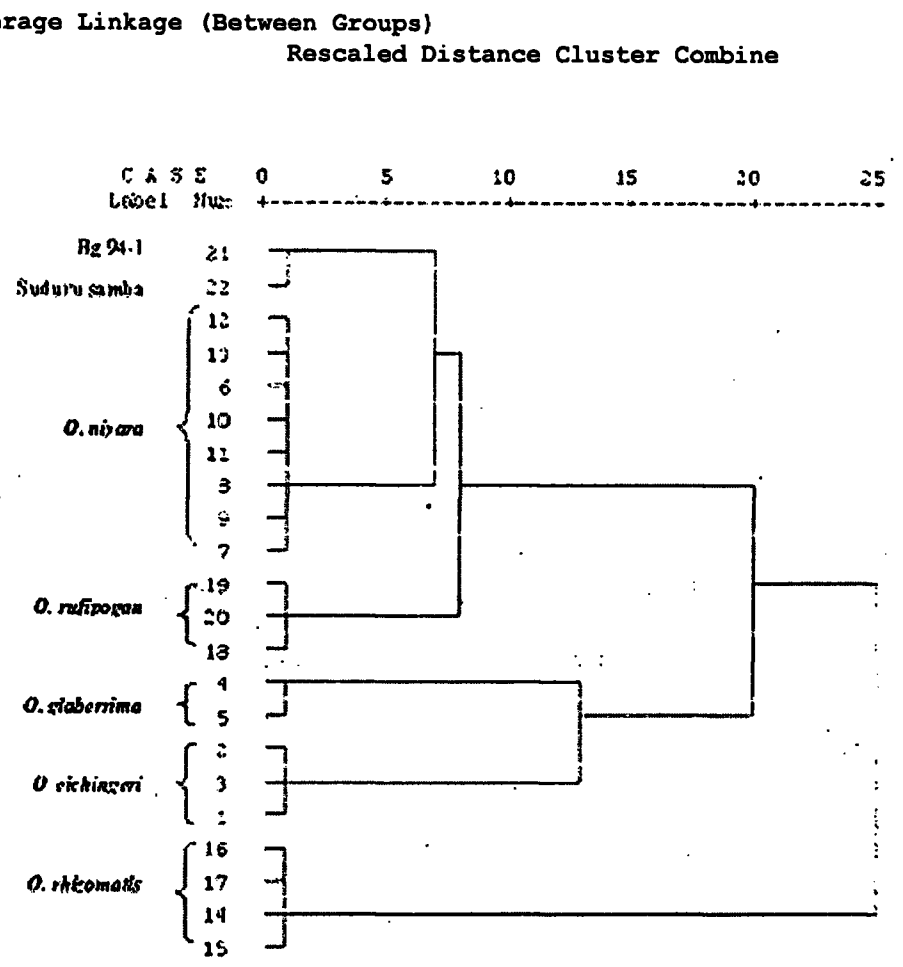

Figure 1. Dendrogram of locally collected wild rice with one introduced *(O. nivara, O. rufipogon, O. eichingeri. O. eichingeri)* wild rice (*O. glaberrima*), cultivated rice(Bg 94-1) and traditional rice(Suduru samba) constructed by cluster analysis of morphological characters.

Cluster analysis of DNA profiles

The dendrogram constructed after analysis of banding patterns generated by all the accessions with ten primers is presented in figure 3. However, three primers did not produce bands for One O. *glaberrima* accession, Code 4. This could have been due to faulty inexact template conditions. The twenty-two accessions taken were clustered into two major groups including genetic distance between as well as within the species. Genomic group belongs to AA *ie. O. sativa, O. nivara, O. rufipogon* and *O. glaberrima* were clustered together while *O. eichingeri* and *O rhizomatis* clustered into same branch revealing genomic group CC. Also *O. glaberrima* was clustered with *O. sativa* indicating their close relationship. *O. glaberrima*

is cultivated rice in African countries. Accessions of *O. nivara*, which showed divergence, are genetically related more closely to *O. rufipogon*. The dendrogram also revealed that *O. nivara* and *O. rufipogon* related to *O. sativa* than the others. There was a high genetic diversity among the individuals of *O. eichingeri* although the collection was very small. *O. rhizomatis* represented a separate cluster.

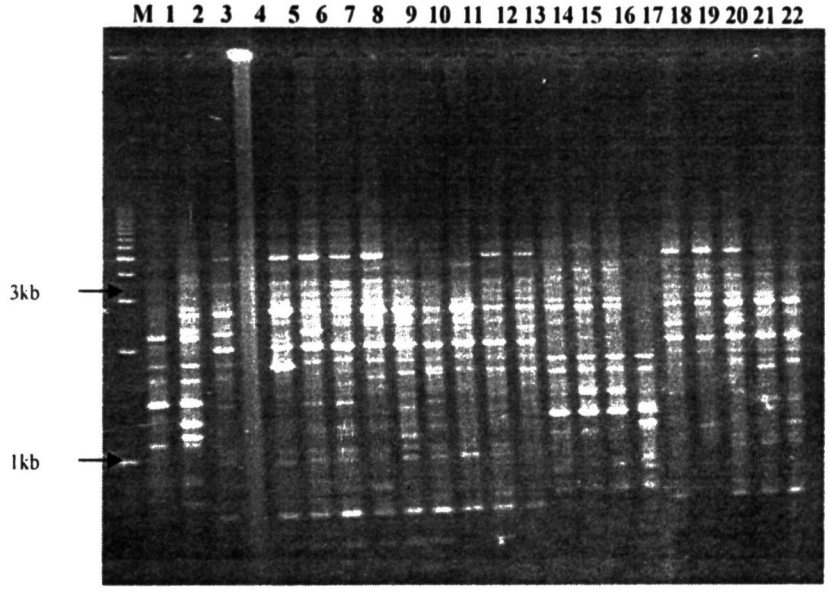

Figure 2. RAPD fingerprints of twenty accessions of wild rice and 2 cultivated rice with primer OPA-20 M-1 kb DNA ladder; 1– 3, *O. eichingeri*; 4 and 5, *O. glaberrima*; 6 – 13, *O. nivara*; 14 – 17, *O. rhizomatis;* 18 – 20, *O. rufipogon*; 21, Bg 94-1; 22 Suduru Samba.

Table 2. Primers and corresponding RAPD markers generated with 22 accessions of wild rice germplasm.

Primer	*Sequence (5'-3')*	*Polymorphic bands*		*Monomorphic bands*	*Total bands*
		Shared	*Unique*		
OPA-20	GTTGCGATCC	14	1	0	15
OPA-17	GACCGCTTGT	21	2	0	23
OPC-04	CCGCATCTAC	17	-	2	19
OPC-15	GACGGATCAG	15	-	1	16
OPC-16	CACACTCCAG	13	4	2	19
OPD-08	GTGTGCGCCA	17	-	3	20
OPD-12	CACCGTATCC	16	1	0	17
OPD-15	CATCCGTGCT	21	2	1	24
OPD-20	ACCCGGTCAC	15	5	1	21
OPK-16	GAGCGTCGAA	19	2		21
TOTAL		168	17	10	195

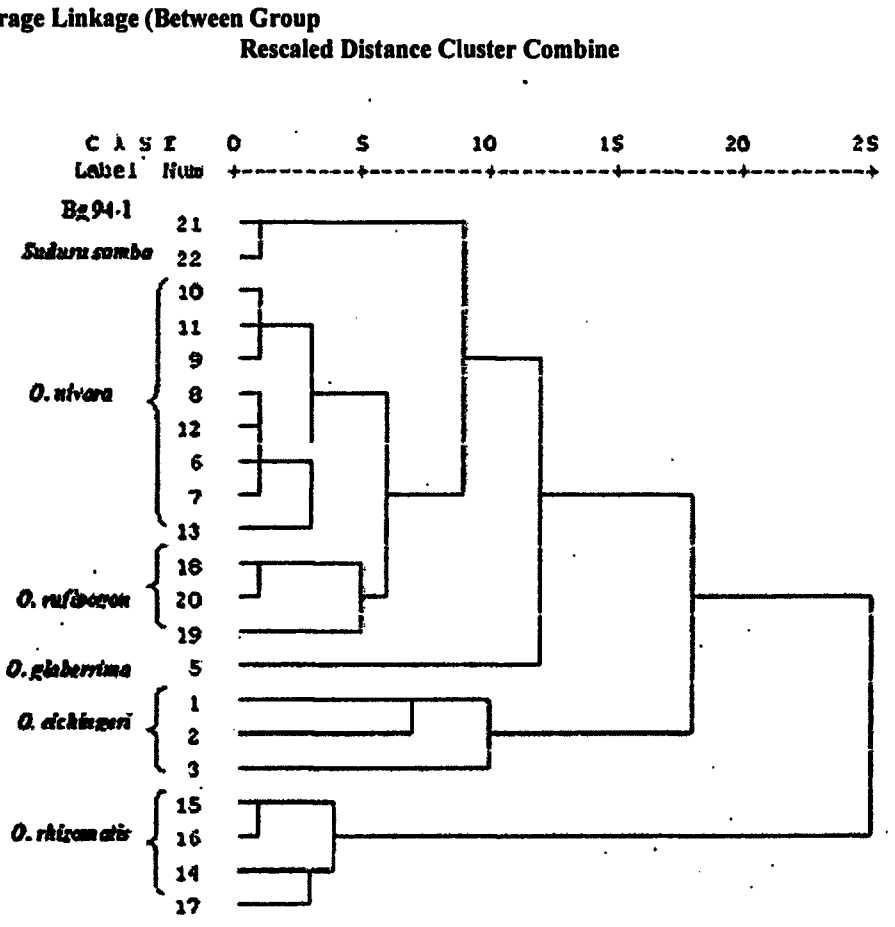

Figure 3. Dendrogram of locally collected *(O. nivara, O. rufipogon, O. eichingeri. O. eichingeri)*, wild rice with one introduced wild rice species (*O. glaberrima)*, cultivated (Bg 94-1) and traditional (Suduru samba) rice constructed by cluster analysis of RAPD markers.

With the development of the polymerase chain reaction (PCR), a new range of molecular technologies become available for the detection, characterization and evaluation of diversity at the genetic level. These techniques vary in the way that they resolve differences, in the type of data that they generate and in the taxonomic levels at which they can be most appropriately applied.

Molecular characterization can reveal the maximum genetic variation or genetic relatedness found in a population (Xu *et al.*, 2000). The standardized RAPD can be successfully used for analyzing genetic diversity (Samarajeewa *et al.*, 2002). If the genetic diversity is high discrimination of the accessions can be done with a lower number of random primers. We have been able to characterize locally collected wild rice germplasm by using 10

random primers. High genetic diversity in the wild rice collection suggests that further collection can be made to enrich gene bank holding of the species.

CONCLUSIONS

The genetic distances suggest the relationship between the species and within the accessions of the same species. There is a high genetic diversity in locally collected wild rice germplasm although the collection was very small. Data generated here is important for further enhancement of the collection.

ACKNOWLEDGEMENTS

The study was supported in full by the SL-USA Cooperative Germplasm Development Program project No 02. The authors are grateful to Dr. R.M.T. Rajapakse for help and suggestions given during this study.

REFERENCES

Amante, A.D., R. de la Pena, L. A. Sitch, H. Leung and T.W. Mew. 1990. Sheath blight (ShB) resistance in wild rices International Rice Research News letter. 15(3):5.

Doyle, J.J. and J.N. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Ikeda, R., G.A.J. Busto and T. Ogawa. 1990. Resistance of wild rice to bacterial blight (BB). International Rice Research News letter. 15(3): 14.

IRRI-International Rice Research Institute 1990 Program report for 1989. Manila, Philippines.

Jones, C.J., K.J. Edwards, S. Castiglione. 1997. Reproducibility testing of RAPD, AELP and SSR markers in plants by a network of European laboratories. Molecular breeding. 43: 23-29.

Khan, Z.R., J.A. Litsinger, A.T. Barrion, F.F.D. Villanueva, N.J. Fernandez and L.D. Taylo. 1991. World bibliography of rice stem-borers. IRRI, Manila, Philippines.415 p.

Fatokun, C.A., D. Danesh, N.D. Young, and E.L. Stewart. 1993. Molecular taxonomic relationships in the genus *Vigna* based on RFLP analysis. Theoory. Appl. Genc. 86:97-104.

Kaga, A., N. Tomooka, Y. Egawa, K. Hosaka and O. Kamijima. 1996. Species relationships in the subgenus Ceratotropis (*Vigna*) as reveal by RAPD analysis. Euphytica, 88: 17-24.

Heinrichs, E.A., F.G. Medrano and H.R. Rapusas. 1985. Genetic Evaluation for insect resistance in rice. IRRI. Manila,Philippines 45-52.

Karp, A., O. Sebery and M. Buiatti. 1996. Molecular techniques in the assessment of botanical diversity. Annals of Botany. 78: 149-149.

Oelke, E.A., T.M.Teynor, P.R. Carter, J.A. Percich, D.M. Noetzel, P.R. Bloom, R.A. Porter, C.E. Schertz, J.J. Boedicker and E.I. Fuller. 1997. Wild Rice. Alternative pp. 1-18.

Samarajeewa, P.K., H.L.V. Nanayakkara, E.M.D.S.N. Ekanayake and V.A. Sumanasinghe. 2002. RAPD analysis of genetic relationships of wild and cultivated *Vigna* species. Annals of the Department of Agriculture Sri Lanka. 4: 215-226.

Vaughan, D.A. 1994. The wild relatives of rice. IRRI, Manila, Philippines.

Virk, P.S., H.J. Newbury, M.T. Jackson and B.V. Ford-Lloyd. 1995. The identification of duplicate accessions within a rice germplasm collection using RAPD analysis Theo. Appl. Gene. 90: 1094-1055.

Williams, J.G., A.R. Kubelic, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research. 18: 6531-6535.

Xu Ru-Qiang, T. Norihiko, A.D. Vaughan and K. Doi. 2000. The *Vigna angularis* complex: Genetic variation and relationships revealed by RAPD analysis and their implications for *In-situ* conservation and domestication. Genetic Resources and Crop Evolution. 47: 123-134.

Yoon, M.S., K. Doi, A. Kaga, N. Tomooka and D.A. Vaughan. 2000. Analysis of genetic diversity in the *Vigna minima* complex and related species in East Asia. J. Plant Resh. 113: 375-386.