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Genetic Analysis of Rice (*Oryza* spp.) Accessions Using Randomly Amplified Polymorphic DNA (RAPD) and Microsatellites

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ABSTRACT. *Assessment of genetic diversity and identification of superior genotypes are important prerequisites for any crop improvement program. The advent of molecular markers detecting variation at the DNA level has opened up new horizons widening the scope for crop improvement. In the present study, 28 rice accessions comprising improved cultivars, landraces and wild Oryza species were screened for genetic diversity using 36 RAPD and 30 microsatellite markers. Out of the total 279 RAPD bands generated, 95 % were polymorphic among the accessions. All of the 52 SSR loci generated were polymorphic. Cluster analysis based on RAPD banding pattern grouped the rice accessions into two major clusters. It also showed that IR62266 was distantly related to O. australiensis and O. officinalis. Cluster analysis based on microsatellite data confirmed this clustering pattern of the accessions. SSR analysis also revealed that IR62266, in addition to O. australiensis and O. officinalis, was distantly related to CT9993 as well. The study identified diverse lines to develop populations for mapping of agronomic traits in rice.*

INTRODUCTION

The genus *Oryza* (family *Poaceae*) comprises 22 wild species and two cultigens (*Oryza sativa* and *Oryza glaberrima*) and provides food for more than one-third of the world's population. It is grown on more than 148 million hectare, in a wide range of ecosystems under varying temperatures, and between 55°N and 36°S latitudes (Khush, 1997). It is grown under diverse hydrological conditions such as irrigated, rainfed lowland, upland and flood prone ecosystems. Human selection and adaptation of rice to diverse environments has resulted in numerous cultivars/varieties. It is estimated that about 120,000 accessions of rice exist in the world today (Khush, 1997). There is a need to study these accessions for their genetic diversity to introduce greater genetic variation in rice improvement programs. The information on genetic diversity was earlier based on morphological characteristics. Morphological traits reflect not only the genetic contributions of the cultivar but also the interaction of the genotype with the environment. Hence, the descriptions based on morphological data are inadequate in providing reliable information.

The developments in DNA marker technology have revolutionized the study of plant populations providing opportunities to address questions requiring a better understanding of variation at the genomic level. Various molecular marker techniques are being practiced in finger printing such as Restriction Fragment Length Polymorphism (RFLP) (Wang *et al.*, 1992), Random Amplified Polymorphic DNA (RAPD) (Virk *et al.*, 1995), Amplified Fragment Length Polymorphism (AFLP) (Vos

et al., 1995) and microsatellites or Simple Sequence Repeats (SSR) (Ramakrishna *et al.*, 1995). Some of these techniques are robust and reliable, e.g., RFLP and AFLP, while some are quick, e.g., RAPD and some others are quick as well as reliable, e.g., microsatellites. But the use of RFLP and AFLP involves radioactive chemicals and therefore, their use is restricted. PCR based markers such as microsatellites and RAPD have been of great use in genetic diversity analysis, genome mapping and gene tagging because they are technically simple, time saving, highly informative and require small amounts of DNA (Thanh *et al.*, 1999). The use of RAPDs for identification of rice accessions was suggested by Fukuoka *et al.*, (1992). Chan *et al.*, (1995) studied the classification of cultivated rice, *Oryza sativa* L. and the common Chinese wild rice, *O. rufipogon* using RAPD. Xie and Zhou (1998) studied the phylogenetic relationship of genus *Oryza* by RAPD analysis. Microsatellites consisting of AT repeats, which are highly polymorphic in rice genome, have been used to distinguish *japonica* cultivars (Akagi *et al.*, 1997). Thanh *et al* (1999) studied the genetic variation in root morphology and microsatellite loci in upland rice accessions from Vietnam. Genetic diversity and population genetic structure of a wild rice (*O. rufipogan* Griff.) in China was studied using microsatellites (Zhou *et al.*, 2003). However, there is little information on the extent of genetic diversity at molecular level among rice accessions grown in different ecological habitats in India. Thus, the study was undertaken with an objective to evaluating the extent of genetic variation at the molecular level among the rice accessions and selected wild *Oryza* species from different ecological habitats.

MATERIALS AND METHODS

The experiment was conducted at Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India during 2001-2002. Several landraces and improved cultivars differing in ecological habitat were chosen for genetic diversity analysis (Table 1). The seeds were obtained from Paddy Breeding Station, Coimbatore and Agricultural Research Station, Paramakudi of this University. Six accessions of wild *Oryza* species (Table 1) also included. The seeds of the wild accessions were obtained from International Rice Germplasm Centre (IRGC), International Rice Research Centre (IRRI), Philippines and West African Rice Development Association (WARDA), Africa.

Isolation of DNA

One to two gram seeds were germinated in the dark using Hoagland's nutrient solution. Fifteen day old etiolated seedlings were used for genomic DNA isolation following standard CTAB extraction procedure (Gawel and Jarret, 1991). The final DNA concentration was adjusted to 25 ng μl^{-1}.

RAPD analysis

Thirty six random primers *viz.*, OPAK 05, 06, 11, 13, 14, 16, 17, 19, OPK 01, 02, 15, 19, OPR 01, 02, 03, 04, 05, 06, 07, 09, 10, 11, 13, 16, OPS 01, 02, 04, 05, 07, 08, 09, 12, 14, 15, 16 and 20 (Operon Technologies Inc., USA) were used to amplify the genomic DNA. All the primers had a GC content of 60-70%. 15 µl of the PCR cocktail contained 25 ng of genomic DNA, 10 mM Tris buffer, 2 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP and dTTP, 6 ng of primer and 0.6 units of *Taq* DNA polymerase. The PCR reaction was performed in a PTC100™ Thermocycler (MJ Research Inc., USA) with a profile of 35 cycles of 1 min at 92°C, 1 min at 37°C

and 1 min at 72°C with initial denaturation for 5 min at 94°C and final extension at 72°C for 7 min. After PCR amplification, 4 µl of 10 X loading buffer was added to the amplified products and then run on 1.5% agarose gel for 4-5 h. at 60-70 V. The gels were stained with ethidium bromide and documented using Alpha Imager 1200 (Alpha Innotech Corp., USA). In the amplified profile, bands of DNA were scored for their presence and absence.

Table 1. Details of rice landraces, cultivated varieties and wild *Oryza* species used.

Accession	Ecotype	Origin	Ecological adaptation
Cultivars			
IR62266	*Indica*	IRRI, Philippines	Lowland
Purpleputtu	*Indica*	TNAU, Coimbatore, India	Lowland
Nootripathu	*Indica*	TNAU,Paramakudi, India	Upland
Varappukodanchan	*Indica*	TNAU, Paramakudi, India	Upland
PMK2	*Indica*	TNAU, Paramakudi, India	Upland
Pokkali	*Indica*	Vyttila, Kerala, India	Saline lowland
ADT38	*Indica*	TNAU, Aduthurai, India	Lowland
ASD16	*Indica*	TNAU, Ambasamudram, India	Lowland
IR50	*Indica*	IRRI, Philippines	Lowland
IR20	*Indica*	IRRI, Philippines	Lowland
IR72	*Indica*	IRRI, Philippines	Lowland
IR64	*Indica*	IRRI, Philippines	Lowland
IR36	*Indica*	IRRI, Philippines	Lowland
Kallurundaikar	*Indica*	TNAU, Paramakudi, India	Upland
TKM11	*Indica*	TNAU, Thiruvurkuppam, India	Upland
TNAU-831311	*Indica*	TNAU, Coimbatore, India	Lowland
W1263	*Indica*	Warangal, Andhra Pradesh, India	Upland
Co43	*Indica*	TNAU, Coimbatore, India	Saline lowland
Norungan	*Indica*	Paramakudi, Tamil Nadu, India	Upland
Mughisali	*Indica*	Assam, India	Upland
Kushal	*Indica*	Assam, India	Upland
CT9993	*Japonica*	CIAT, Columbia	Upland
Wild species	***Genome***	**Source**	
O. nivera	AA	IRGC, Phillippines	Upland
O. rufipogan	AA	IRGC, Phillippines	Lowland
O. spontania	AA	IRGC, Phillippines	Lowland
O. glaberrima	AA	WARDA, Africa	Upland
O. officinalis	CC	IRGC, Phillippines	Lowland
O. australiensis	EE	IRGC, Phillippines	Saline lowland

Microsatellite analysis

Thirty microsatellite primers *viz.*, RM 1, 5, 6, 14, 16, 17, 19, 20, 23, 24, 29, 35, 36, 44, 50, 55, 60, 71, 80, 81, 83, 107, 124, 148, 160, 164, 167, 209, 210 and 327 (Rice Map Pairs, Research Genetics Inc., USA) were used. 15 µl of the PCR cocktail

contained 25 ng of DNA, 10 mM Tris buffer, 250 µM each of dATP, dCTP, dGTP and dTTP, 1.0 µl each of both forward and reverse primers and 0.6 units of *Taq* DNA polymerase. The PCR reaction had 35 cycles of 1 min at 94°C, 30 sec at 57°C, 1 min at 72°C with initial denaturation for 2 min at 95°C and final extension at 72°C for 5 minutes. After amplification, 4 µl of loading dye was added to the amplified products after denaturation at 95°C and the products were run on a sequencing apparatus (Amersham Pharmacia, USA) using 6% urea-polyacrylamide gels. The gels were run at 60 watts till the dye reached the bottom of the gel. Silver staining of the gels was done to visualize the amplified products (Andrews, 1986). The gels were dried and transferred to Whatman 3 mm filter papers. The bands were recorded for the presence or absence.

Construction of dendrogram

The amplified products were scored separately for each RAPD and microsatellite primer for landraces, cultivated varieties and wild species. Presence and absence of individual bands was denoted as 1 and 0, respectively. The scores of individual bands were used to create a data matrix as described by Rohlf (1990). The similarity index (SI) values were computed as a ratio of number of similar bands to the total number of bands in pair wise comparison of the genotypes. A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) with unweighted pair group method (UPGMA) (Sokal and Michener, 1958) using the NTSYS-pc version 2.02 (Exeter Software, New York, USA).

RESULTS AND DISCUSSION

RAPD analysis

A total of 279 scorable RAPD bands were generated among 28 rice accessions in the present study. Of which 95% were polymorphic among the accessions. The RAPD products generated had a molecular weight ranging from 500 to 2000 bp. RAPD profile of one representative primer is shown in Plate 1. The maximum number of amplified products were generated by the primers OPS04 (12) followed by OPAK 19 (11) and OPR11 (11) that are having the GC content varied from 60-70% and this was in accordance with the findings made by Williams *et al.* (1990). They found that primers with GC content more than 40% to give maximum polymorphism. In the case of rice, it is found that the number of amplicon products tended to increase with increasing GC content in the range between 40 and 60% (Fukuoka *et al.*, 1992).

Cluster analysis using RAPD data

A dendrogram was generated using data matrix from RAPD primers (Figure 1). It was seen that the 28 rice accessions were grouped into two major clusters namely, A and B. The major Cluster A comprised of 26 accessions, except *O. australiensis* and *O. officinalis*, which were grouped in Cluster B.

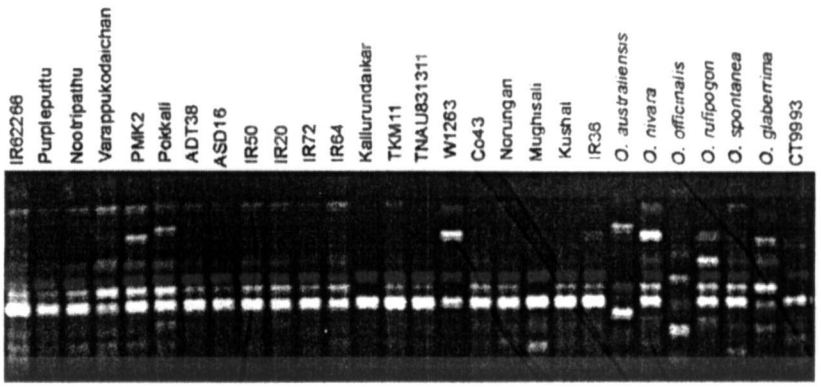

Plate 1. RAPD profile generated for 28 rice accessions using primer OPR03.

The major Cluster A was divided into two minor Clusters namely, A_1 and A_2. The minor Cluster A_1 consisted of 25 accessions except *O. glaberrima,* which was grouped, in A_2. Cluster A_1 was further divided into two sub clusters namely A_3 and A_4. Cluster A_3 consisted of 22 accessions. Cluster A_4 had Kallurundaikar, W1263 and Mughisali.

Within the major Cluster A, all the improved cultivars *viz.*, IR20, IR72, IR64, IR50, IR36, Co43 and ADT38 were grouped together in A_5. These are high yielding cultivars normally suited to irrigated lowland ecosystem. In the same cluster, the wild species namely, *O. nivara* and *O. spontanea* were also grouped. This may be because these wild species also have AA genome as in the case of *O. sativa* accessions. However, within major Cluster A, two wild species have been grouped separately. The sub cluster A_2 consisted of *O. glaberrima.* A_6 comprised *O. rufipogon.* Based on the dendrogram, it is evident that all the accessions having the AA genome were grouped together in the major Cluster A, while Cluster B contained two wild species, *O. australiensis* and *O. officinalis* with different genomes. *O. australiensis* has EE genome, while *O. officinalis* has CC genome. This separate grouping of the wild species may be because they are quite divergent from the cultivated accessions at genomic level (Aggarwal *et al.,* 1999; Singh *et al.,* 1999). Wild species are sources of biotic and abiotic stress tolerance genes and have been used in wide crosses for introgression of resistance genes into the cultivated accessions (Jena and Khush, 1990; Brar and Khush, 1997).

Based on the SI, it can be seen that within the major Cluster A, IR62266 is most distantly related to Kallurundaikar and W1263. This may be because IR62266 is a lowland accession from Philippines, while Kallurundaikar is an upland accession from Tamil Nadu, India and W1263 is an upland accession from Andhra Pradesh, India. The geographical isolation and ecological habitat of these accessions may have led to divergence at the molecular level. Among the different *Oryza* species IR62266 is most distantly related to *O. australiensis* and *O. officinalis.* Such discrimination among accessions has been reported in rice (Zheng *et al.,* 1991; Yu and Nguyen, 1994; Mackill, 1995; Xie and Zhou, 1998; Linu *et al.*, 2000).

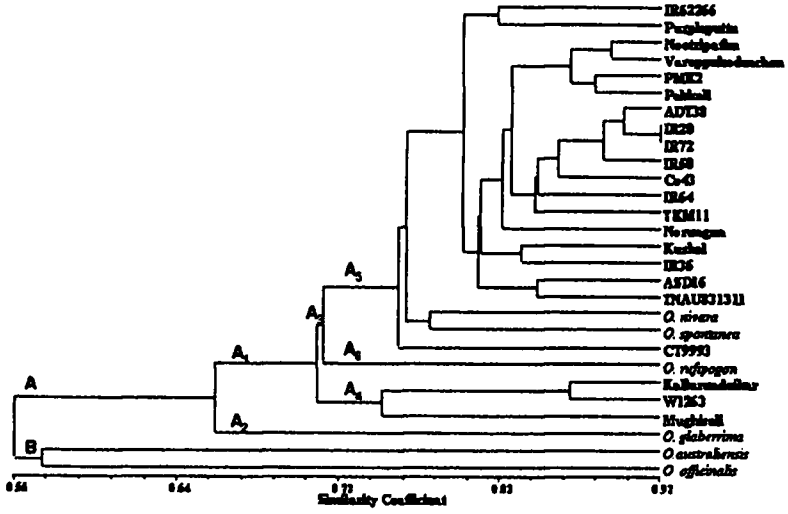

Fig. 1. Dendrogram for 28 rice accessions based on similarity coefficient matrix from RAPD primers.

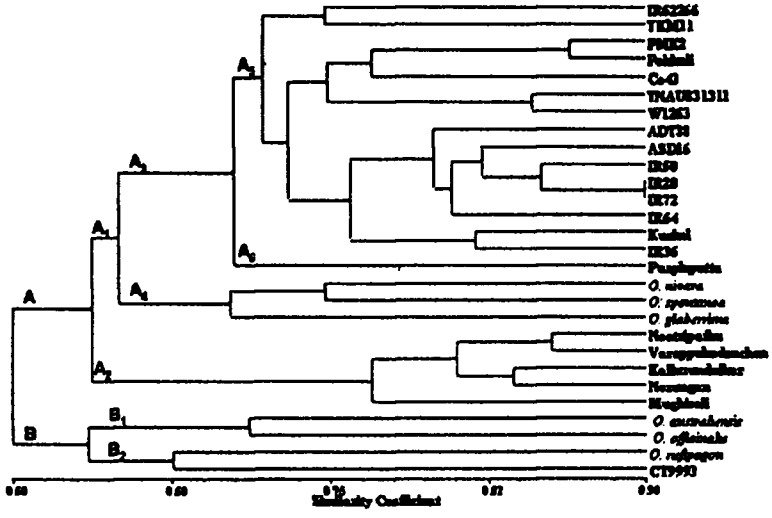

Fig. 2. Dendrogram for 28 rice accessions based on similarity coefficient from 30 microsatellite primers.

SSR analysis

A total of 52 SSR alleles were generated among 28 rice accessions. All of them were polymorphic. The size of different alleles amplified varied from 70-400 base pairs. The average number of alleles per microsatellite locus was found to be

3.66. The number of alleles per primer pair varied from 2 to 9. The banding pattern of one SSR primer is shown in Plate 2.

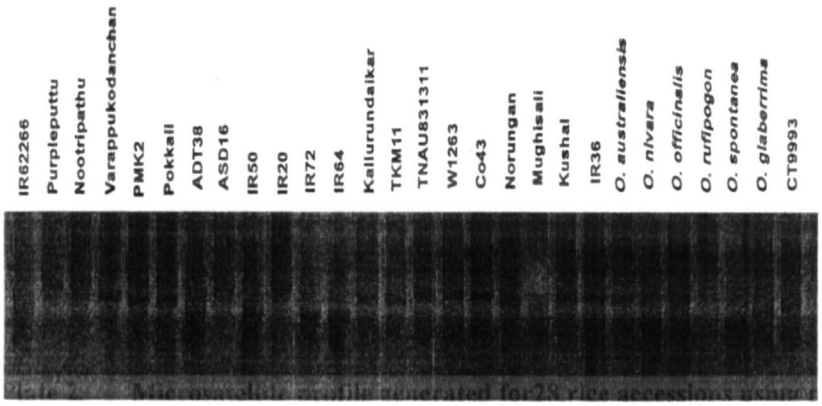

Plate 2. Microsatellite profile generated for28 rice accessions using primer RM 164.

Cluster analysis using SSR primers

The dendrogram constructed for the 28 accessions based on the SI values generated using 30 SSR primers is shown in Figure 2. As in the case of RAPD, the accessions have been grouped into two major clusters namely, A and B. The major cluster A comprised 24 accessions. Cluster B comprised CT9993, *O. australiensis, O. officinalis* and *O. rufipogon.*

All the improved accessions were grouped together in sub Cluster A_5. In the same sub cluster, Co43 and Pokkali show a similarity of 78% at the molecular level which is justified because both these accessions are salt tolerant. In minor Cluster A_2, the landraces viz., Nootripathu, Varappukodanchan, Kallurundaikar and Norungan were grouped together which reflects their close similarity at the molecular level. These landraces are from rainfed upland ecosystem of Tamil Nadu, India.

The wild accessions are grouped in two separate clusters namely, Cluster A_4 and Cluster B. Cluster A_4 consists *O. nivara, O. glaberrima* and *O. spontanea.* This may be because they all have AA genome. In the major Cluster B, the minor Cluster B_1 has *O. australiensis* and *O. officinalis* which have different genomes from all the other accessions included in the study. Cluster B_2 had *O. rufipogon* and CT9993 and both of them have AA genome.

On comparing, the *O. sativa* subspecies within the major Cluster A, it is evident that IR62266 was most distantly related to Kallurundaikar. This may be because IR62266 is from Philippines irrigated lowland, while Kallurundaikar is a landrace from rainfed uplands of Tamil Nadu. However, on comparison of all the accessions within the major Cluster A, IR62266 was most distantly related to *O. spontanea.*

On interclustral analysis, it was seen that IR62266 and CT9993 were the most distantly related with only 47.8% similarity among themselves. This may be because IR62266 is a lowland *indica* accession from Philippines, while CT9993 is a *japonica* upland accession from Columbia.

Comparison of RAPD and SSR cluster analysis

On comparing the dendrograms derived using RAPD and SSR data, the one based on SSR data brought out the genetic diversity among the rice accessions more clearly. Though both dendrograms had grouped the accessions into two major clusters, Cluster B in the SSR based tree delineated the accessions with genome other than AA, CT9993, a *japonica* with AA genome and *O. rufipogon,* which is most distantly related to *AA* genome wild species (Brar and Khush, 1997). In RAPD derived tree all the accessions with AA genome were grouped together in the major Cluster A. In SSR derived dendrogram, the landraces within the major Cluster A were grouped separately in A_2 while this was not the case in the RAPD tree.

CONCLUSION

Both RAPD and SSR markers were able to distinguish the genetic diversity among the selected rice accessions at the molecular level. However, SSR derived dendrogram gave more information on divergence among the accessions based on dissimilarity at genomic level than RAPD derived one and so SSR can be used for future studies on genetic diversity. This may be because they are locus specific, co-dominant and reproducible. This study identified lines that can be used in rice improvement program.

REFERENCES

Aggarwal, R.K., Brar, D.S., Nandi, Huang, N. and Khush, G.S. (1999). Phylogenetic relationships among *Oryza* species revealed by AFLP markers. Theor. Appl. Genet. 98: 1320-1328.

Akagi, H., Yokaseki, Y., Inagaki, A. and Fujumura, J. (1997). Microsatellite DNA markers for rice chromosomes. Theor. Appl. Genet. 93: 1071-1077.

Andrews, A.T. (1986). Electrophoresis: Theory, techniques and biochemical and clinical applications. Oxford University Press, Oxford, UK.

Brar, D.S. and Khush, G.S. (1997). Alien introgression in rice. Plant Mol. Biol. 35: 35-47.

Chan, C.Y., Young, C.T., Howan, P. and Seo, S.H. (1995). Genetic polymorphisms and phylogenetic relationship of Korean rice based on RAPD markers. Korean J. Breed. 278: 86-93.

Fukuoka, S., Hosaka, K. and Kamijima, O. (1992). Use of Random Amplified Polymorphic DNA (RAPD) for identification of rice accessions. Jpn. J. Genet. 67: 243-252.

Gawel, N.J. and Jarret, R.L. (1991). A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea* plants. Mol. Biol. Rep. 9 : 262-266.

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. Bull. Soc. Sci. Nat. 44: 223-270.

Jena, K.K. and Khush, G.S. (1990). Introgression of genes from *Oryza officinalis* Well ex Watt to cultivated rice, *O. sativa* L. Theor. Appl. Genet. 87: 737-745.

Khush, G.S. (1997). Origin, dispersal, cultivation, and variation of rice. Plant Mol. Biol. 35: 25-34.

Linu, M., Babu, R.C., Souframanien, J., Chezhian, P., Shanmugasundaram, P., Nagarajan, P. and Sadasivam, S. (2000). DNA polymorphism among rice (*Oryza sativa* L.) accessions differing in drought tolerance. J. Plant Biol, 27(2): 1-8

Mackill, D.J. (1995). Classifying *japonica* rice cultivars with RAPD markers. Crop Sci. 35: 889-894

Ramakrishna, W., Chowdari. K.D., Lagu, M.D., Gupta, V.S. and Ranjekar, P.K. (1995). DNA fingerprinting to detect genetic variation in rice using hypervariable DNA sequence. Theor. Appl. Genet. 90: 1000-1006.

Rohlf, R.J. (1990). NTSYS-PC, numerical taxonomy and multivariate analysis system, version 1.8, Exeter Software, Setauket, New York.

Singh, K.N., Nandi, R., Shanmugasundaram, P., Sadasivam, S., Huang, N., Brar, D.S. and Khush, G.S. (1999). High-resolution DNA fingerprinting of Indian rice (*Oryza sativa* L.) varieties by amplified fragment length polymorphism. Genet. Res. Crop Evo. 46: 427-433.

Sokal, R.R. and Michener, C.D. (1958). A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38: 1409-1438.

Thanh, N.D., Zheng, H.G., Dong, N.V., Trinh, L.N., Ali, M.L. and Nguyen, H.T. (1999). Genetic variation in root morphology and microsatellite DNA loci in upland rice (*Oryza sativa* L.) for Vietnam. Euphytica. 105: 43-51.

Virk, P.S., Newbury, H.J., Jackson, M.T. and Ford-Lloyd, B.V. (1995). The identification of duplicate accessions within a rice germplasm collection using RAPD analysis. Theor. Appl. Genet. 90: 1049-1055.

Vos, P., Hogers. R., Bleeker, M., Raijans, M., Vandelee, T. and Homes, M. (1995). AFLP a new technique for DNA finger printing. Nucleic Acids Res. 23: 4407-4414.

Wang, Z.Y., Second, G. and Tanksley, S.D. (1992). Polymorphic and phylogenetic relationship among species in the genus *Oryza* as determined by analysis of nuclear RFLPs. Theor. Appl. Genet. 83: 565-581.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: 6531-6535.

Xiao, J., Li, J., Yuan, L., McCouch, S.R. and Tanksley, S.D. (1996). Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. Theor. Appl. Genet. 92: 637-643.

Xie, Z.W. and Zhou, Y. (1998). Phylogenetic relationship of genus as revealed by RAPD analysis International Rice Research Newsletter. 23(3): 6-8.

Yu, L.X. and Nguyen, H.T. (1994). Genetic variation detected with RAPD markers among upland and lowland rice cultivars (*Oryza sativa* L.). Theor. Appl. Genet. 87: 668-672.

Zheng, K.L., Shen, B. and Qian, H.R. (1991). DNA polymorphisms generated by arbitrarily primed PCR in rice. Rice Genetics Newsletter. 81: 134-136.

Zhou, H.F. Xie., Z.W. and Ge, S. (2003). Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogan* Griff.) in China. Theor. Appl. Genet. 107: 332-339.