Nordic Journal of Botany 29: 238–243, 2011
doi: 10.1111/j.1756-1051.2011.00998.x,

Subject Editor: Anne Krag Brysting. Accepted 14 January 2011

Genetic differentiation among Sri Lankan traditional rice (*Oryza sativa*) varieties and wild rice species by AFLP markers

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Traditional rice varieties are one important component of the biodiversity of Sri Lanka. However, no proper studies have been performed on genetic diversity of the Sri Lankan traditional rice varieties used in our breeding programs. In the present study, the genetic diversity of 46 traditional rice (*Oryza sativa*) varieties and 5 wild rice species is assesed using Amplified Fragment Length Polymorphism (AFLP) markers. Ten primer combinations generated a total of 784 fragments. Of these, 772 fragments were polymorphic (98.4%). UPGMA analysis based on Jaccard's similarity separated the accessions into four major clusters. A cophenetic correlation with $r = 0.786$ strongly supported the clustering pattern of UPGMA dendrogram. A principal coordinate analysis (PCoA) also confirmed the UPGMA clusters. Accessions referred to the same cluster showed similar morphological characteristics (e.g. height, grain colour etc.) while accessions which are known to be morphologically distinct appeared genetically separated. In addition, the clustering pattern distinctly separated lowland and upland rice varieties. This genetic diversity assessment at the molecular level provides reliable information for selection of germplasm in the development of new rice varieties and in conservation of traditional rice genetic resources.

Oryza sativa varieties which have been grown in Sri Lanka from ancient time to the middle of the last century are known as traditional rice varieties. Sri Lanka is considered as one of the secondary diversity centers for rice genetic resources (Ikeda and Vaughan 1991). In ancient times, farmers cultivated traditional rice varieties, because of their adaptability to Sri Lankan soil types, climate, geography and harsh environmental conditions such as flood, drought, soil salinity, iron toxicity, pests and diseases. With the introduction of high yielding improved varieties, traditional varieties became extinct from cultivation (Morishima and Oka 1995). However, most of the old varieties are still conserved ex-situ as seeds in gene banks.

Genetic diversity or phylogenetic relationships are very useful when selecting parents for hybridization to develop new cultivars with desirable characteristics such as tolerance to biotic and abiotic stress. Such information permits rice breeders to select genetically diverse parents to obtain desired traits when developing new varieties. Understanding the association of characters is of prime importance in selecting parents and screening progeny for new varietal developments. In traditional breeding these relationships have been studied based on pedigrees (Oakey et al. 2006), morphological characteristics (Khalid et al. 2010), sexual compatibility etc. (Bernacchi and Tanksley 1997). It has been shown that, compared to morphological studies and pedigree analysis, molecular markers reveal differences among accessions at genomic level and thus provide more direct and reliable information for varietal differentiation (Ni et al. 2002). Conventional selection methods of parental varieties based on morphology are time consuming and dependent on environmental factors. Breeding a new variety based on phenotypic characters may take more than a decade and even then the release of an improved variety cannot be guaranteed. Molecular markers make this procedure more efficient and expedite the selection process in rice breeding. Hence, molecular markers are widely used as an efficient tool to characterize genetic variability. Among various marker systems, the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) is a robust and highly informative DNA fingerprinting method to assess a large number of markers without prior sequence knowledge. As a result, AFLP has been widely used to study different organisms including bacteria (Lin et al. 1996), fungi (Cirvilleri et al. 2007), plants (Ercisli et al. 2009), nematodes (de Gruijter et al. 2006) and mammals (Cameron et al. 2003) where much is still unknown about the genomic makeup. AFLP is widely accepted as an effective tool for identification of genomic differences among closely related species (Loh et al. 1999).

The objective of this study was to assess the genetic diversity among different traditional rice varieties and wild

rice species available in the Gene Bank of Sri Lanka by AFLP markers and thereby provide genetic diversity information for conservation and efficient use of rice germplasm collection in new varietal developments.

Material and methods

Plant material

The rice varieties/accessions used in this study are listed in Table 1. These are broadly classified into four groups: traditional rice varieties (TR), wild rice species (WS), modern rice varieties (MV) and *Hygroryza aristata* (closely related genus to genus *Oryza*). Seeds were obtained from the seed bank of Plant Genetic Resources Centre (PGRC) at Gannoruwa, Peredeniya, Sri Lanka. The seeds (10–15 of each variety) were planted in pots of the same size filled with paddy soil and grown under greenhouse conditions without adding fertilizers. After two weeks the young tender leaves were harvested from 3–4 randomly selected plants from each variety/accession, and stored at −80°C until extraction of DNA.

Extraction of DNA

Genomic DNA was extracted from rice leaves using a CTAB based method as described by Chen and Ronald (1999). The extracted DNA was quantified by Agarose gel electrophoresis followed by visualization under the UV transilluminator with standard DNA concentration markers. Then concentrations of all DNA samples were adjusted to 300 ng μl^{-1}.

AFLP analysis

AFLP analysis was performed as described by Vos et al. (1995) with minor modifications such as changes in amounts of DNA, PCR components and cycle conditions. Briefly; 1 μg of DNA from each sample was digested with 5 units of *Eco*R1 and 5 units of *Mse*1 enzymes. The digestion sample was incubated at 37°C for 3.5 h.

To the double digested DNA, *Eco*R1 adaptor (10 pmol μl^{-1}), *Mse*1 adaptor (10 pmol μl^{-1}), 5U of T4 DNA ligase were added and incubated overnight (~16 h) at 37°C. After ligation of adaptors, 5 μl of digested/ligated DNA were preamplified in 25 μl of reaction containing 20 pmol of each preamplification primers, 0.5 mM dNTPs, 1U of Taq DNA polymerase (5U μl^{-1}), 1× PCR buffer containing 1.5 mM $MgCl_2$. The PCR amplification was performed with the following temperature profile: 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 60 s. The preamplification product was diluted 20× with sterile distilled water and used as a template for selective amplification (sequences of the adaptors, preamplification primers and selective amplification primers are shown in Table 2).

The selective amplification reaction was conducted in a final volume of 20 μl containing 5 μl of diluted preamplification product, fluorescently labeled (HEX or TMR or FAM) *Eco*R1 primer (5.6 pmol), *Mse*1 primer (5.6 pmol), 0.5 mM dNTPs, 1U of Taq DNA polymerase (5U μl^{-1}), 1× PCR buffer containing 1.5 mM $MgCl_2$. Selective primer combinations and fluorescent tags used are shown in Table 3. The PCR amplification was carried out as follows. Denaturation at 94°C for 30 s, annealing at 58°C for 30 s in the first cycle and then the annealing temperature was reduced by 0.7°C per cycle for the next

Table 1. List of traditional rice varieties and wild rice species used in this study. TR = traditional rice, WS = wild rice species, MV = modern variety.

No.	Accession no.	Name	Type	No.	Accession no.	Name	Type
1	5642	Pokkali	TR	27	2340	Wedaheenati	TR
2	3142	Molaga-samba	TR	28	6276	Mudukiriel	TR
3	3445	yakada-wee	TR	29	7799	SuduheenatiIcpy19	TR
4	3470	Hatada-wee	TR	30	4724	Goda al wee	TR
5	3689	Kiriyal	TR	31	2056	Gonabaru	TR
6	3707	Heenati	TR	32	3147	Kaluheenati	TR
7	3725	Siviru wee	TR	33	3598	Bala mawee	TR
8	3131	Dahanala	TR	34	3355	Suduheenati1cpy15	TR
9	3136	Pachchaiperumal	TR	35	6248	Kombili	TR
10	3143	Sulai 27614	TR	36	6233	Kalu kura wee	TR
11	3145	Podiwi A 8	TR	37	4278	Kohumawi B11	TR
12	3150	Vellaiperunel	TR	38	2199	Suwada samba	TR
13	3151	Vellai Ilankalayan	TR	39	3177	Madael	TR
14	3156	Oddavalan	TR	40	3724	Goda heenati	TR
15	3183	Hathiel	TR	41	8499	Hondarawala	TR
16	3484	Kuruluthudu wi	TR	42	6188	Heen dik wee	TR
17	3133	Murunga137	TR	43	4992	Rathu heenati	TR
18	3444	Dikwee	TR	44	3751	Chembavala samba	TR
19	3478	Kahata wee	TR	45	4614	Chembavala samba	TR
20	4832	Hatapanduru wee	TR	46	2079	Murunga	TR
21	4909	Niyan wee	TR	47	6290	Taichung Native1	MV
22	5630	Peillianel 26081	TR	48	10030	*O. rhizomatis*	WS
23	6169	Dewaradderi 26081	TR	49	5138	*O. rufipogon*	WS
24	6283	Murunga kayan	TR	50	10096	*O. nivara*	WS
25	3727	Kottamalli	TR	51	10031	*O. eichingeri*	WS
26	5569	Red leaf variety	TR	52	10199	*O. granulata*	WS
				53		*Hygroryza aristata*	

Table 2. Sequence of the adaptors, preamplification primers and selective amplification primers used for the AFLP analysis.

Adapters and primers	Sequence
Adaptors	
*Mse*1 adapter	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
*Eco*R1 adapter	5′-CTGCTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
Pre amplification primers	
E00 primer	5′-GACTGCGTACCAATTC- 3′
M00 primer	5′-GATGAGTCCTGAGTAA-3′
Selective amplification primers	
*Eco*R1 primer	5′-GAC TGC GTA CCA ATT CNN-3′
*Mse*1 primer	5′-GAT GAC TCC TGA GTA AAA N-3′ N-selective nucleotide

12 cycles. Then the cycles were repeated 23× with the annealing temperature of 49°C. Extension was carried out at 72°C for 1 min for all cycles.

The amplified samples were purified by ethanol precipitation followed by washing with 70% ethanol. The dried pellets were re-suspended in 5 µl of water and 2.5 µl of sample was mixed with ET550-ROX size standard and deionized formamide according to the manufactures instructions. Then the samples were denatured at 95°C for 2 min and analyzed by capillary electrophoresis on an automated MegaBACE 1000 DNA sequencer. AFLP fragment analysis was performed with Genetic Profiler software ver.2.2.

Data analysis

Peaks in the range of 30–550 bp were analyzed and compared by using the MegaBACE Genetic Profiler software ver. 2.2. Each sample was analyzed and compared in triplicates and unambiguous, reproducible peaks in the electropherogram were scored as AFLP markers whereas fragments appearing inconsistent in the triplicates were excluded. A tolerance limit of 1.0 base and a minimum peak height of 100 were consistently maintained while scoring the peaks. Each differently sized fragment was treated as a unique character and converted to binary data as presence (1) and absence (0).

Jaccard's similarity coefficients (Jaccard 1901) were calculated using binary data and a similarity coefficient matrix was generated to assess the genetic resemblances among varieties. Then the similarity matrix was subjected to cluster analysis by the unweighed pair group method with arithmetic mean (UPGMA) method as suggested by Sneath and Sokal (1973) and the dendrogram was generated by multi-variate statistical package MVSP 3.1 software (Kovach 1998). The confidence of the UPGMA clusters were assessed by a Mantel test (Mantel 1967) to calculate the cophenetic correlation coefficient (r) as described in Dighe et al. (2004). The cophenetic correlation coefficient is a comparison between the dendrogram and the similarity matrix.

The genetic similarity between the different accessions was also calculated by the Dice coefficient as $GS_{xy} = 2a/(2a+b+C)$ where GS_{xy} is the measurement of the genetic similarity between accessions x and y, a is the number of polymorphic bands present in both accessions, b is the number of polymorphic bands present only in accessions x, and c is the number of polymorphic bands present only in accessions y (Dice 1945) and this similarity coefficient matrix was used for UPGMA cluster analysis by the WINBOOT software (Yap and Nelson 1996). Nei and Li's similarity coefficients (Nei and Li 1979) were also computed and UPGMA cluster analysis was performed using the NEIGHBOR program in PHYLIP ver. 3.69 (Felsenstein 2009). The resulting dendrogram was plotted by the TREEVIEW program (Roderic 1996). All analyses resulted in similar clustering patterns and only the results obtained of the Jaccard coefficient is presented in this paper.

A principal coordinate analysis (PCoA) was performed between the accessions using the Gower general similarity coefficient (Gower and Legendre 1986) to find out possible relations that could not be visualized in the cluster analysis. In the PCoA analysis, data were plotted on first three dimensions using the MVSP software (Kovach 1998).

Table 3. Selective amplification primer combinations and percentage of polymorphism for each primer.

Primer combination	Fluorescent tags used	Total no. of bands	No of polymorphic bands	Polymorphism (%)
E-AA×M-C	TMR	83	81	97.5
E-AA×M-G	HEX	41	40	97.5
E-AC×M-T	HEX	62	62	100.0
E-AG×M-A	HEX	54	53	98.1
E-AG×M-T	HEX	59	59	100.0
E-AT×M-A	HEX	75	72	96.0
E-AT×M-C	HEX	60	59	98.3
E-AT×M-G	HEX	171	168	98.2
E-AT×M-T	HEX	90	89	98.8
E-CA×M-G	FAM	89	89	100
	Total	784	772	98.4

Results and discussion

Level of polymorphism

Sixteen *Eco*R1 and *Mse*1 selective amplification primer combinations were initially screened but only ten pairs of primers which generated good amplification and distinct polymorphism with all 46 rice varieties were selected for data analysis. Those ten pairs of primers generated a total of 784 fragments. Of these, 772 were polymorphic (98.4%). The fragments ranged in size from 30 to 550 base pairs. The number of amplification products generated by individual primer pairs ranged from 41 (E-AA×M-G) to 171 (E-AT×M-G) with an average of 78.4 fragments per primer pair. Eight out of ten primers pairs generated more than 60 fragments. Percentage of polymorphism ranged from 97.5 (E-AA×M-C, E-AA×M-G) to 100% (E-AC×M-T, E-AG×M-T, E-CA×M-G) with an average of 98.4% (Table 3).

Genetic relationship and cluster analysis

All fragments (784) scored were used for the genetic diversity analysis. Because all analyses methods resulted in similar clustering patterns, only the results obtained by

Jaccard's coefficient are discussed. The pairwise Jaccard's similarity between accessions ranged from 0.065 to 0.494. The varieties Heendikwee and Siviruwee showed the lowest genetic similarity (0.083) while the varieties Weedaheenati and Mudukiriel showed the highest genetic similarity (0.494). The average genetic similarity between all accessions (0.209) was used to establish a cut-off value for a cluster generation. At this cut-off value, UPGMA analysis separated the 46 Sri Lankan traditional rice varieties into four main clusters (Fig. 1). Cluster I and cluster IV contained only one variety each while cluster II and III comprised 26 and 25 varieties/ accessions, respectively.

Hygroryza aristata, which has previously been referred to *Oryza*, was introduced in the analysis as an outlier. As expected, *H. aristata* was found to be the most divergent line and clustered in cluster I by separation from all other accessions at the similarity coefficient value of 0.127. Most of the varieties found the same cluster also show similar morphological characteristics (e.g. height, grain colour etc.). Cluster II contained 26 accessions including 5 wild rice

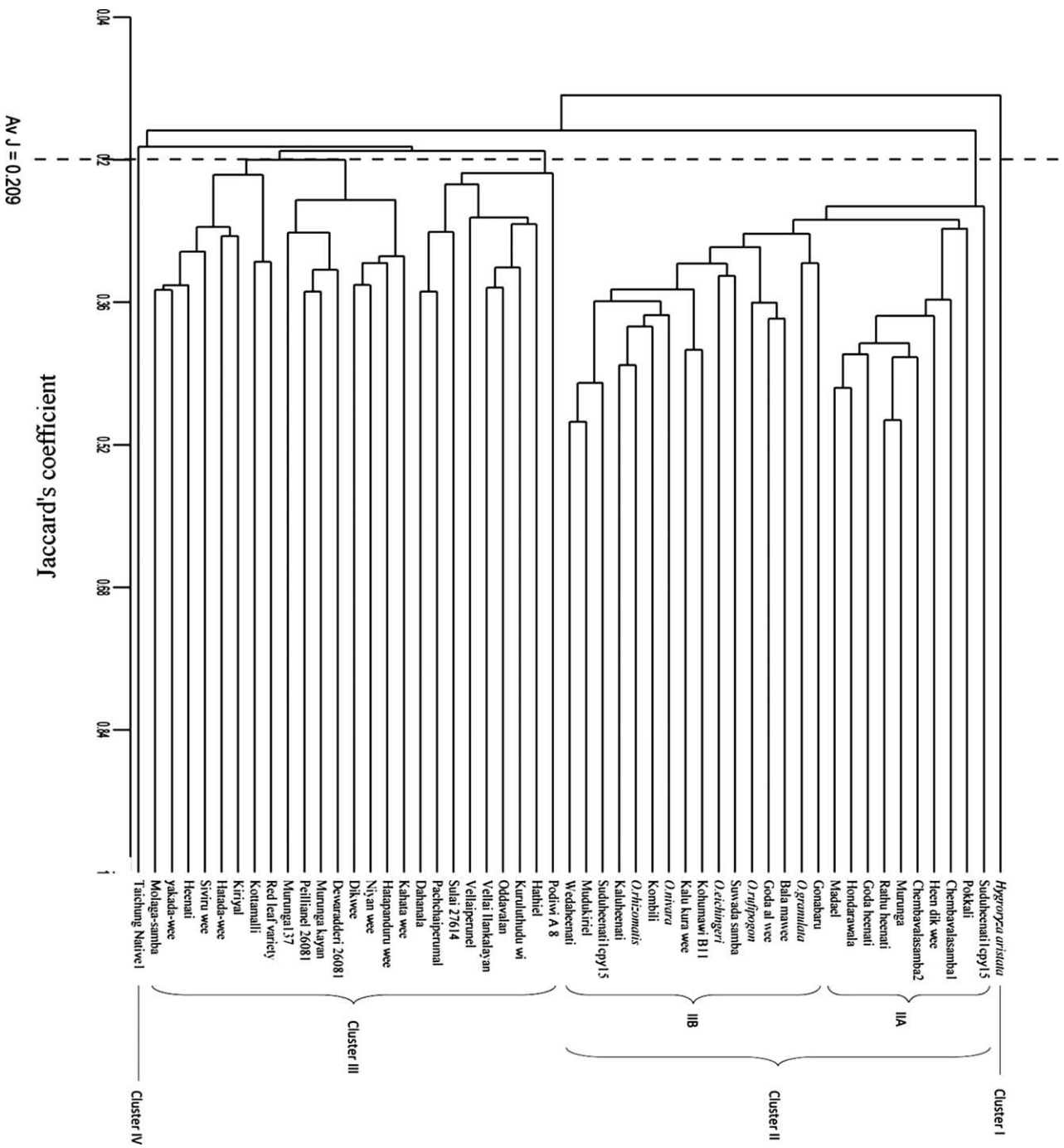

Figure 1. The UPGMA dendrogram showing genetic diversity among Sri Lankan traditional rice varieties based on Jaccard's similarity coefficient. AVJ = average genetic similarity.

species. This cluster was again subdivided into two subclusters (IIA and IIB) at the similarity coefficient of 0.345. Two different accessions (acc. 3751 and acc. 4614) from the same variety 'Chembavala samba' were found in cluster IIA. All five wild species *Oryza nivara, O. rufipogon, O. rhizomatis*, *O. granulata* and *O. eichingeri* turned up in cluster IIB. Morphologically very similar wild species *O. nivara* and the traditional variety 'Kombili' are derived from the same branch with a high genetic similarity coefficient (0.372). Furthermore, cluster II and cluster III contained lowland and upland varieties, respectively. Life-span for the lowland varieties was 3–6 months while it was below 4 months for upland varieties. Cluster IV contained the only modern variety used in this study called Taichung Native 1(TN-1), which is the first *Indica* variety carrying the well known Dee-geo-woo-gene (semi dwarfing gene), and is more susceptible to pest and diseases and is photoperiod-insensitive. Cluster III enclosed 25 traditional varieties and many of them are believed to have originated in India and subsequently grown in Sri Lanka as traditional varieties (e.g. Molaga Samba, Pachchaperumal, Vellaiperunel, Vellai Illankalayan, Peillinel and Murunga). These plants were cultivated in northern part of Sri Lanka during 1930's.These results confirm the reliability of data and analysis methods. For further verification of data, a UPGMA dendrogram was constructed (Fig. 2) using AFLP data only for five wild rice species. According to the dendrogram, wild species *O. rhizomatis* which has been reported only from Sri Lanka (Vaughan 1989) and the species *O. eichingeri* were derived from the same node. Both species were found to be close relatives or in between types (Zhang and Ge 2006). Although their habitats are distinctly different, Sri Lankan *O. eichingeri* is sympatric to *O. rhizomatis* with their population being overlapping in both northern and southern Sri Lanka (Bautista et al. 2006). Moreover, both *O. eichingeri* and *O. rhizomatis* have the CC genome (Bautista et al. 2006). These similarities are clearly seen in the dendrogram. Furthermore *O. nivara* and *O. rufipogon* which are genetically similar with the AA genome (Yosuke et al. 2007) are separated from *O. granulata* with the GG genome (Toshie et al. 2007). *Oryza granulata* with the GG genome did not group into any other species. Therefore this dendrogram reiterated the consistency of AFLP data and analysis method.

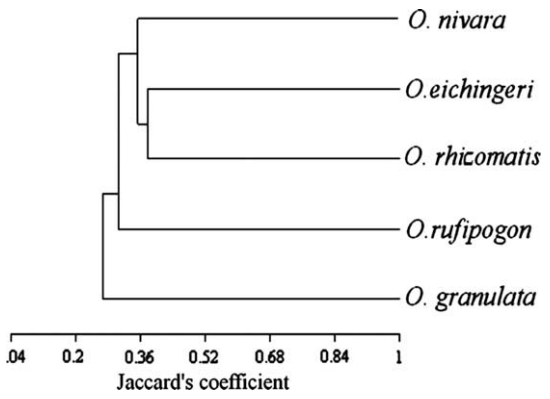

Figure 2. Dendrogram based on Jaccard's similarity coefficient for five wild rice species using AFLP analysis.

The cophenetic correlation coefficient (r) indicated how well the UPGMA dendrogram represented similarity data. High values of r strongly support the clustering pattern of dendrograms. The r value for UPGMA analysis of Sri Lankan traditional rice varieties (Fig. 1) was 0.798 and it was 0.957 for the dendrogram, which contains only five wild rice species (Fig. 2). This indicated a very good fit of similarity data to groups in the cluster analysis.

Figure 3 shows a principal coordinate analysis (PCoA) of 53 rice genotypes based on AFLP data obtained from the similarity matrix constructed by Gower general coefficient. The distribution of groups produced by the PCoA analysis confirms the clustering pattern of the UPGMA analysis. A scatter diagram of first three coordinates (PCo1, PCo2 and PCo3) revealed three well defined groups of varieties. All wild rice species were found among the varieties in group A which corresponded to the cluster II of the dendrogram (Fig. 1) while group B and group C correspond to the cluster III of the dendrogram showing very similar differentiation of varieties as represented by the UPGMA analysis. Furthermore the high genetic divergence of TN1 from other varieties was also displayed.

Conclusions

This is the first report on genetic analyses of Sri Lankan traditional rice varieties by fluorescent AFLP markers. The AFLP results presented here clearly indicated that the genetic diversity observed among the traditional rice varieties is significantly high. *Hygroryza* used as an outlier, separated on its own, confirm its identity as a separate genus. TN1 with some unique characters separated from the rest as an individual. Lowland rice varieties demanding different agronomic practices separated from the upland varieties. Wild species grouped in the same cluster along with some accessions indicate close genetic relatedness. All methods of analyses produced similar results confirming the reliability of data used in this study. Therefore, the genetic diversity information obtained will be useful in efficient use of Sri Lankan rice germplasm collection. In addition, this information will be useful in management of in situ and ex situ germplasm collections in conservation programs for traditional rice varieties.

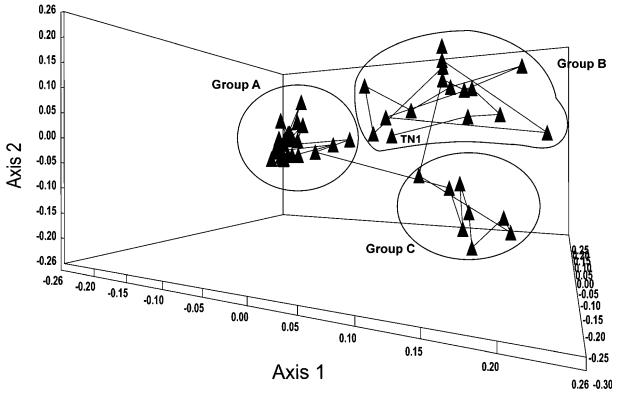

Figure 3. Scatter diagram of the first three principal cordinates (PCo1, PCo2 and PCo3) of 46 traditional rice varieties based on AFLP markers. Ovals indicate well-defined groups.

Acknowledgements – We thank the National Research Council of Sri Lanka (grant no. 05-61) for financial support. Technical assistance by Ms Anoma Jayasoma is gratefully acknowledged.

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