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Full Length Research Paper

Contrasting genetic diversity among *Oryza longistaminata* (A. Chev et Roehr) populations from different geographic origins using AFLP

D. K. Kiambi[1]*, H. J. Newbury[2], B. V. Ford-Lloyd[2] and I. Dawson[3]

[1]International Plant Genetic Resources Institute (IPGRI) – Regional Office for sub-Saharan Africa,P.O. Box 30677, Nairobi, Kenya.
[2]School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.
[3]International Center for Research in Agroforestry (ICRAF), P.O. Box 30677, Nairobi, Kenya.

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Molecular markers have been used extensively in studying genetic diversity, genetic relationships and germplasm management. However, the understanding of between and within population genetic variation and how it is partitioned on the basis of geographic origin is crucial as this helps to improve sampling efficiency. The objective of this study was therefore to assess the intra-specific diversity in Oryza longistaminata and how the variation is partitioned within and between different geographic locations, using molecular markers. AFLP analysis generated 176 bands that revealed high levels of polymorphism (95.6%) and diversity within and between populations. The mean Nei's genetic diversity for all the 176 loci in the 48 populations was 0.302 and diversity for populations within countries ranged from 0.1161 to 0.2126. Partitioning of between and within population diversity revealed that the mean allelic diversity at each polymorphic locus was HT = 0.3445. The within population diversity was (HS = 0.1755) and the between population diversity was (DST = 0.1688). Results of AMOVA revealed significant differences ($p<0.05$) in genetic variation among populations within different countries of the region. Genetic parameters estimated from AFLP data indicated that there are high levels of genetic diversity in the wild populations of *O. longistaminata* studied and that this diversity is higher within than between populations. Hierarchical partitioning also revealed that most of this diversity is found between populations within countries than among countries. Regional collection and conservation strategies therefore need to consider country differences while national strategies should consider population differences within countries.

Key words: Molecular markers, AFLP, Oryza longistaminata, populations, genetic diversity.

INTRODUCTION

The knowledge of the extent and structure of genetic variation in populations of wild plants is essential not only for understanding the processes of evolution but also the development of appropriate and efficient strategies for the collection and conservation of populations (Frankel and Bennet, 1970). The wild relatives of cultivated rice are an important constituent of the rice gene pool and have contributed significantly to breeding programmes. Evaluation of the populations continues to reveal new sources of resistance to diseases and pests. Interest in these species has increased as techniques to transfer genes from wild species to cultivated rice improve (Heinrichs et al., 1985; Khush et al., 1990; Brar and Khush, 1995). *O. longistaminata* A. Chev. et Roehr is

*Corresponding author. Tel.1-514-398-7851. Fax.1-514-398-7879. E-mail: dionysious.kiambi@mail.mcgill.ca.

one of the wild rice species that is already making significant contributions to rice improvement programmes, particularly in Asia and Africa (Khush et al., 1990; Jones et al., 1996).

Molecular markers have a lot of potential for application in genetic diversity studies and the management of plant genetic resources as clearly shown by Virk et al. (1995; 2000), Ford-Lloyd et al. (1997) and Song et al. (2003). In particular, the use of AFLP in studying the rice gene pool is promising as they have proved to be reliable, produce a large number of bands and are more reproducible than RAPDs (Sensi et al., 1996; Xu et al., 1995; Virk et al., 2000). Using AFLPs, Mackill et al. (1996) generated more polymorphic bands than through any other molecular marker method previously used on rice. Cho-YongGu et al. (1996), Nandi et al. (1997) and Virk et al. (1998) have also used AFLP markers in cloning and mapping QTL. Zhu et al. (1998), Raven (1999) and Aggarwal et al. (2002) have clearly demonstrated their efficiency and reliability in analysis of genetic diversity of rice populations.

O. longistaminata belongs to the AA genome group of the sativa complex and hybridizes easily with the cultivated species, *O. glaberrima* and *O. sativa* (Bezançon et al., 1977; Vaughan, 1989). The species is allogamous, has a high rate of self-incompatibility and is thus predominantly outcrossing (Ghesquiere, 1987; Jones et al., 1996). Several studies based on morphology, cytogenetics, ecology and biochemical and molecular markers have significantly contributed in complementary ways to the current knowledge of genus Oryza (Vaughan, 1994). However, the understanding of between and within population genetic variation and how it is partitioned on the basis of geographic origin is crucial as this will help to improve sampling efficiency in order to have a wider representation of the *O. longistaminata* genetic diversity conserved. The objective of this study was therefore to assess the intra-specific diversity in *O. longistaminata* and determine how the variation is partitioned within and between different geographic locations, using the East and Southern African countries as a case study.

We analyzed AFLP markers to study the partitioning of diversity among and within countries, between and within populations using 48 bulked populations and 24 individuals from 8 populations as working samples. The material represented a wide geographical range.

MATERIALS AND METHODS

Plant material

Seed was collected from Kenya (KEN), Zimbabwe (ZIM), Mozambique (MOZ) and Tanzania (TZA) in collaboration with the national programmes. More seed and leaf samples originating from Botswana (BOT), Namibia (NAM), Mozambique, Madagascar (MAD) and Zambia (ZAM) were obtained from the International Rice Gene **B**ank in IRRI. Leaf material was harvested fresh, frozen and stored at -70°C. 48 populations of *O. longistaminata* were selected for analysis to represent the geographical range of the species. For material received as seeds, young leaves were harvested from 2-week-old seedlings of each accession for DNA extraction.

DNA extraction and AFLP analysis

The protocol for isolation of genomic DNA developed by Gawel and Jarret (1991) was followed with modifications, using 20 mg of leaf material from 5-10 seedlings. AFLP analysis was carried out using the methodology of Virk et al. (1998), as modified from Vos et al. (1995). EcoRI from Pharmacia and MseI from New England Biolabs, Inc. were used to restrict the genomic DNA by incubating the mix for 1 h at 37°C followed by ligation to EcoRI and MseI adaptors by incubation at 37°C for 3 h.

Pre-amplification was carried out using EcoRI-(EP; 5'-GACTGCGTACCAATTCA-3') and MseI-(MP; 5'-GATGAGTCCTGAGTAAC-3') adapter-directed primers each having a single selective base (Vos et al., 1995). It was run for 2 h in a Hybaid Omnigene Thermocycler and the products then diluted 50 times. Amplification was carried out using four primers from Operon Technologies Inc. The EcoRI primers possessed three selective bases at the 3' end and were end-labeled with [[γ-33P] ATP using T4 polynucleotide kinase (TPNK–Pharmacia). The amplification was performed using Gene Amp PCR System 9700 and the following profile: one cycle of 95°C; one cycle of 94°C for 1 min., 65°C for 1 min., 72°C for 1 min followed by eight cycles with a stepwise reduction of 1°C in the annealing temperature to 57°C; 27 cycles of 94°C for 30 seconds, 56°C for 1 min, 72°C for 1 min. The reaction was stopped by adding 25 µL of formamide loading buffer containing 98% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol and 1 mg/ml bromophenol blue. The samples were denatured at 90°C for 1 min and 2 µL of the mix was loaded on to a 5% denaturing polyacrylamide gel. The gel was cast in a Sequigen 38x50 cm gel apparatus (Bio-Rad) and electrophoresis run for 2 h at a constant temperature of 50°C. It was dried, exposed to a Kodak Biomax film for 4-5 days and then processed in a COMPACT X2 X-OGRAPH .

Data analysis

Since AFLP are usually dominant markers, only two character states were scored: presence (1) or absence (0) of bands. An assumption was made that the co-migrating fragments in different populations are allelic and map to similar genomic regions (Waugh et al., 1997; Virk et al., 1998, 2000).

Data analysis using PC-ORD Version 4.10 was performed with each primer combination individually. Correlation between distance matrices generated using Jaccard's coefficient was tested with the standardized Mantel Statistics and a significance value assigned. Cluster analysis to show the relatedness of the populations according to the geographic origin (different countries) was performed and a dendrogram generated using the UPGMA algorithm of POPGENE Version 1.31. Jaccard's co-efficient and Sequential Agglomerative, Hierarchical and Nested Clustering (SAHN) feature of NTSYS-pc Version 2.0 were used to generate dendrograms showing the clustering of the 48 populations and the 24 individuals from 8 populations. Principal component analysis was performed using the DECENTER and EIGEN procedures of NTSYS-pc Version 2.0 and both 3-D and 2-D scatterplots generated.

An analysis of molecular variance (AMOVA; Excoffier et al., 1992) was performed on a Euclidean distance matrix generated and analyzed using the ARLEQUIN 1.1 software package. Significance values were assigned to variance components based on the

Table 1. Significance of among countries' population differences.

	MAD	ZAM	BOT	KEN	TZA	ZIM	NAM
ZAM	+						
BOT	-	-					
KEN	+	+	-				
TZA	+	+	-	+			
ZIM	-	-	-	+	+		
NAM	+	+	-	+	+	+	
MOZ	+	+	-	-	+	+	+

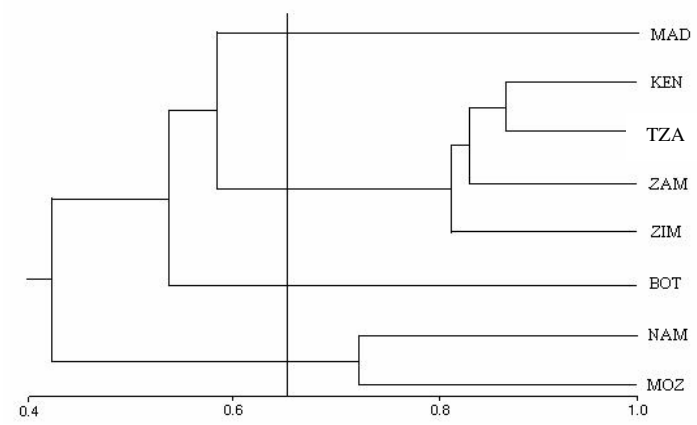

Figure 1. Dendrogram of the relationships between countries based on 48 populations using the UPGMA algorithm method of POPGENE Version 1.31.

random permutation (10,000 times) of individuals assuming no genetic structure. The significance of pair wise Fst values between countries was calculated to check for significant differences. Canonical discrimination analysis of POPGENE 1.31 was used to reveal a 2-dimensional view of relationships between countries by representing the multivariate data on orthogonal axes. POPGENE Version 1.31 was used to calculate genetic diversity in each country based on AFLP product frequencies. At each polymorphic locus, the total allelic diversity is represented by HT, which is partitioned into the mean allelic diversity within populations, HS and the allelic diversity among populations, DST. These quantities are related by the expression HT = HS + DST. The proportion of total allelic diversity found among populations, GST was calculated as the ratio DST/HT (Hartl, 1987; Buso et al., 1998).

RESULTS

The four primers used (E1+M2 and E2+M1) in AFLP analysis revealed a total of 184 bands that were 95.6% polymorphic. The 176 polymorphic bands ranged in size from 24 to 726 bp and clearly distinguished all the 48 populations. 88 polymorphic bands clearly distinguished between the 24 individuals in 8 populations. The value of the standardized Mantel Statistic, r, was 0.82 ($P<0.0002$) indicating a good correlation between primer combinations.

Between country relationships

From the pair wise difference distance method of AMOVA, there were significant differences ($p<0.05$) of populations among all the countries except BOT, as shown in Table 1.

The results of cluster analysis using an arbitrary similarity defined as 1- distance of 65% divided the countries into four major groups that) to a large extent, reflect their geographic origin (Figure 1). One group consisted of MOZ and NAM populations. The second group comprised the BOT populations while the third group revealed an affinity between TZA, ZAM, ZIM and KEN populations.

The fourth group comprised MAD populations, which is quite distinct from the other groups and separates at a similarity of 57%. The mean genetic distances between countries are given in Table 2.

Cluster and PCA analyses were performed in order to visualize the affinities of the 48 populations more clearly. An arbitrary similarity level of 48% was used to define the 48 populations into 6 clusters (Figure 2). Genetic similarities ranged from 32% to 84%. The clustering generally reflected both the geographic origin and levels of diversity, as determined by the Nei's genetic diversity indices.

Cluster 1 comprised MOZ and NAM populations with 14 sub-clusters ranging from 52 to 84% in similarity. Cluster 2 comprises 2 of the 3 MAD populations with similarity of 79%. The cluster is quite distinct, separating from the other populations at 39% similarity. The third cluster comprises TZA populations, which separate from the other populations at 49%. Cluster 4 comprises some populations from TZA, ZIM, ZAM and KEN and separates from the other populations at 52% similarity. Cluster 5 is a unique accession from ZAM, which separates from the other populations at 42%. Cluster 6 comprises TZA, ZAM, ZIM, KEN, BOT and MAD

Table 2. Mean (Nei, 1973) genetic distance between countries.

	MAD	ZAM	BOT	KEN	TZA	ZIM	NAM	MOZ
MAD	****							
ZAM	0.1540	****						
BOT	0.2654	0.1408	****					
KEN	0.1900	0.0549	0.1786	****				
TZA	0.1569	0.0491	0.1665	0.0459	****			
ZIM	0.1872	0.0724	0.2129	0.06436	0.0846	****		
NAM	0.2824	0.2502	0.3622	0.3195	0.2774	0.2862	****	
MOZ	0.2126	0.2030	0.3700	0.2467	0.2192	0.2237	0.1299	****

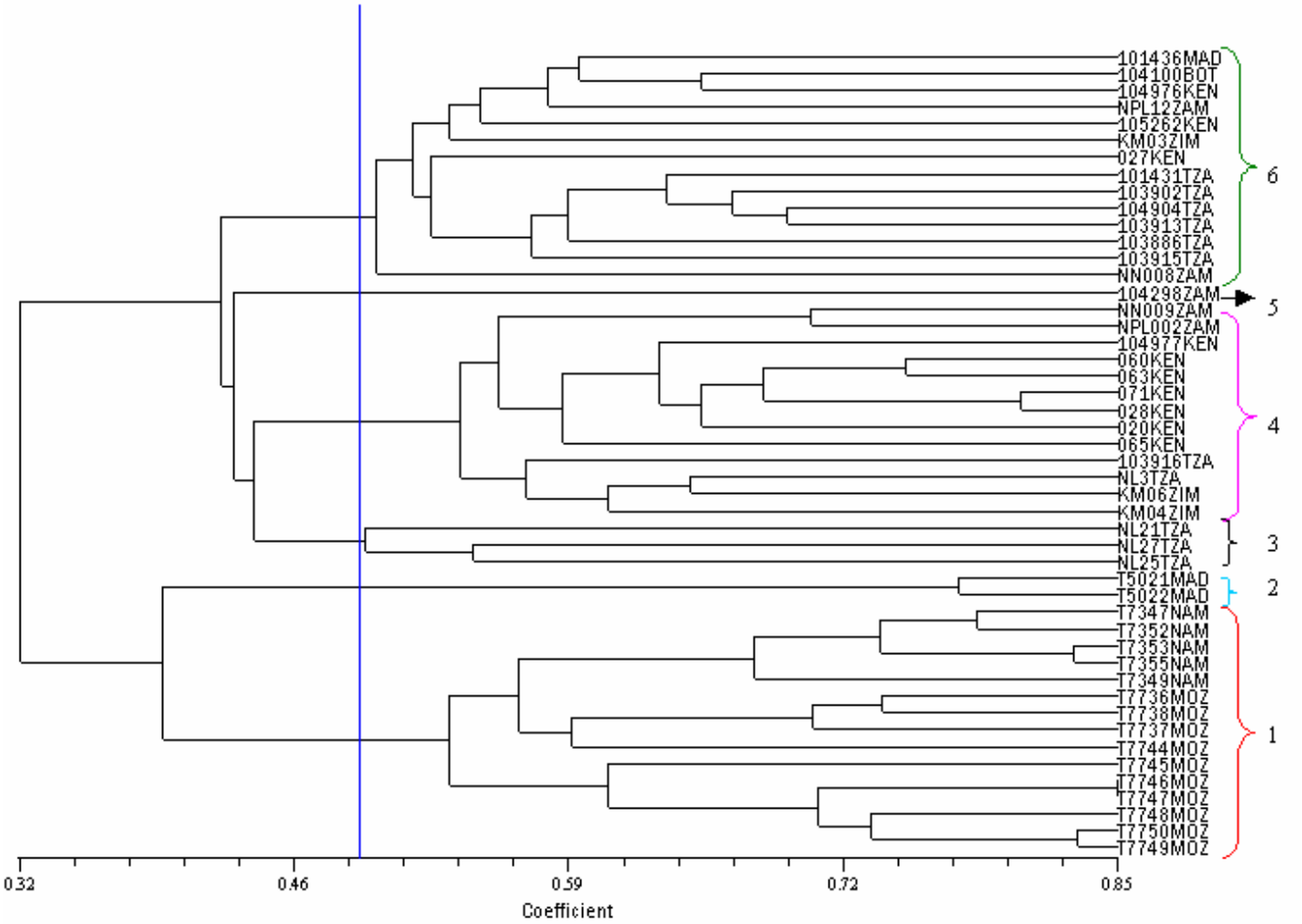

Figure 2. Clustering of 48 populations using 176 polymorphic AFLPs, based on NTSYs-pc UPGMA cluster analysis (Rohlf, 1993) and Jaccard's coefficient.

populations. It separates at 49% similarity and is the most diverse cluster. The TZA and ZAM populations are spread over 3 out of the 6 clusters while KEN, MAD and ZIM populations are found in 2 clusters. NAM and MOZ populations are only found in 1 cluster. The spread of the clusters seems to reflect the within country diversity as indicated by Nei's diversity index.

When the PCA was performed, the first three eigenvectors explained 35.1% (1st 19.4%, 2nd 9% and 3rd 6.7%) of the variation. The 1st principal component separates Cluster 1 comprising MOZ and NAM populations. It also separates Cluster 2 comprising the two MAD populations. The 2nd principal component separates Clusters 4 and 6. The 3rd principal component

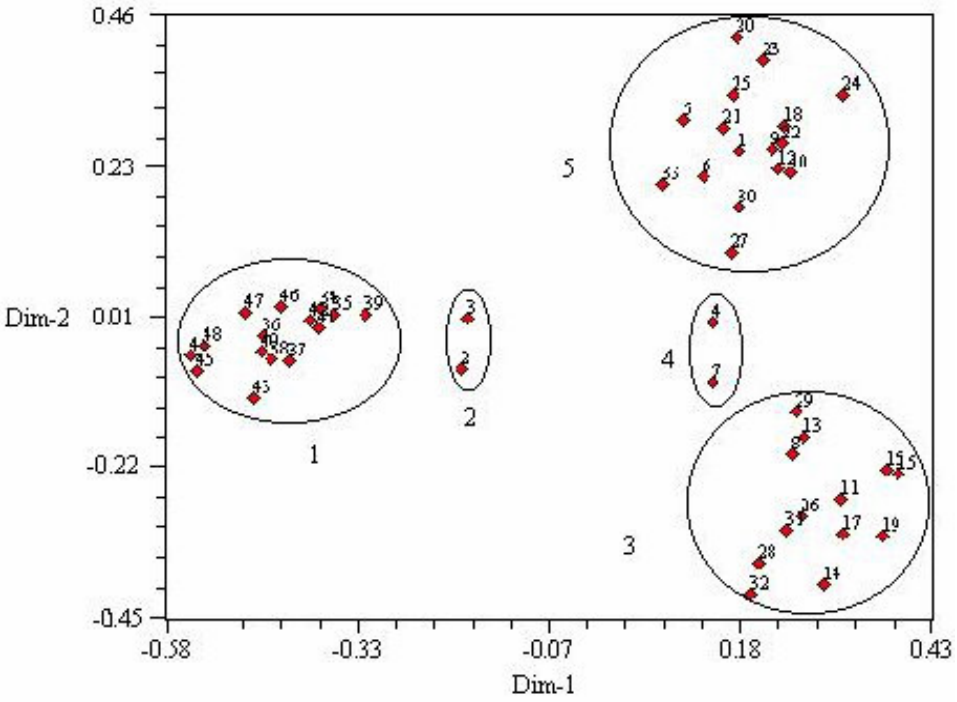

Figure 3. 2-D principal component analysis based on genetic relationships of 48 populations of *O. longistaminata* using the DECENTER and EIGEN features of NTSYS-pc (Rohlf, 1993).

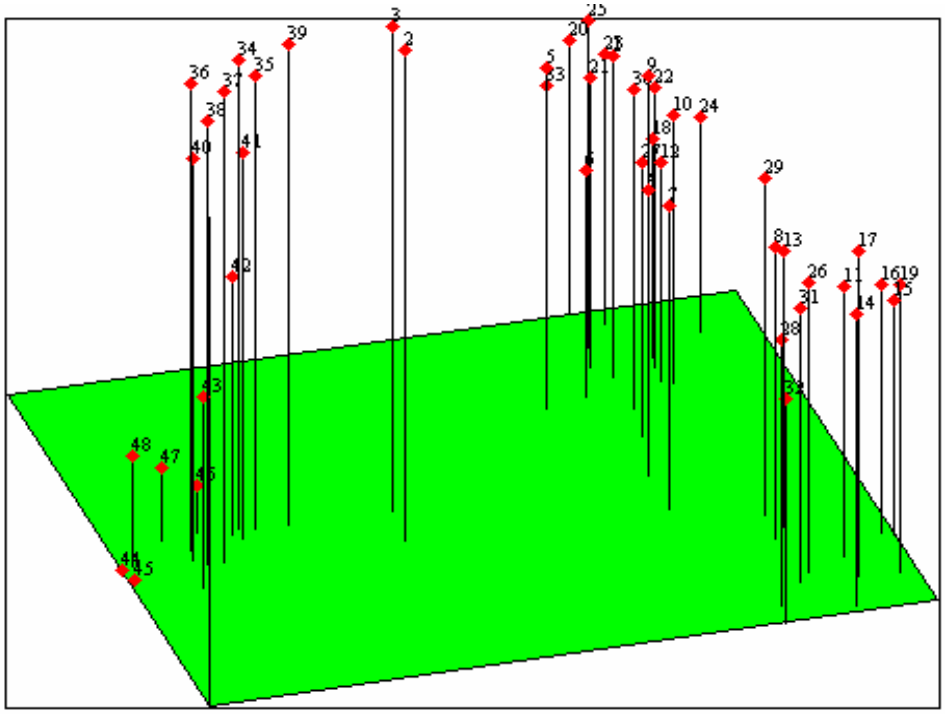

Figure 4. 3-D principal component analysis based on genetic relationships of 48 populations of *O. longistaminata* using the DECENTER and EIGEN features of NTSYS-pc (Rohlf, 1993).

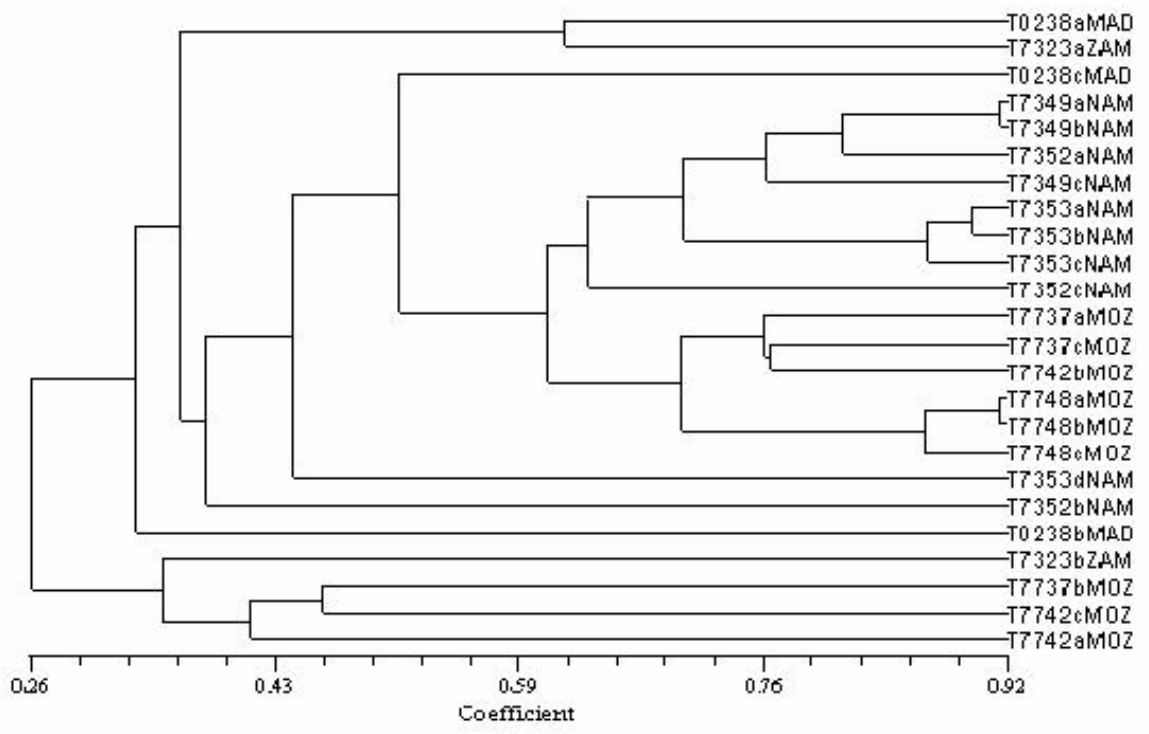

Figure 5. Clustering of 24 individuals from 8 populations using 88 polymorphic AFLPs, based on UPGMA cluster analysis (Rohlf, 1993) using Jaccard's coefficient.

(3-D) clarifies the separation of Cluster 2 and further separates Cluster 1 into MOZ and NAM populations with almost all the MOZ located much closer to the basement. Cluster 5 with one ZAM accession has been separated by the 2nd Principal component though not very distinctly. Cluster 3, having 3 TZA populations, has not been clearly separated by either 3-D or 2-D as shown in the scatter plots provided in Figures 3 and 4.

The visual grouping of 24 individuals from 8 populations using PCA and cluster analysis, indicated that 6 clusters of individuals generally reflecting the diversity were formed at an arbitrary similarity level of 40% as shown in Figure 5. The PCA revealed that the first three eigenvectors explained 49% (1st 28 %, 2nd 12 % and 3rd 9%) of the variation. The analysis shows that the individuals are generally clustering on the basis of the high within population genetic diversity as reflected by Nei's diversity index. The most diverse populations (T7737MOZ and T7352NAM) with diversity indices of 0.268 and 0.257 respectively had the individuals spread out widely in the clusters. The analysis of similarities within populations revealed high within population diversity.

Genetic diversity

There was a clear genetic differentiation both among countries and populations within countries using significance tests based on 10,100 permutations. The examination of the hierarchical partitioning of genetic variation by AMOVA demonstrated that genetic differentiation amongst countries was significant at $p<0.05$ with a variance component of 9.75. The within countries differentiation was significant at $p<0.05$ with variance components of 23.14. Of the total diversity, 29.65% was attributed to country differences while 70.35% of the total variation was attributed to population differences within the countries and the FST value was 0.30.

The mean Nei's (1973) genetic diversity for all the 176 loci in the 48 populations was 0.302. Nei's (1973) mean genetic diversity for populations within countries ranged from 0.1161 to 0.2126. The highest mean diversity was found in TZA (H = 0.2126) followed by ZAM and MAD. The lowest diversity was found in NAM (H = 0.1161) followed by MOZ, ZIM and KEN. The partitioning of between and within population diversity using 24 individuals from 8 populations revealed that the mean allelic diversity at each polymorphic locus was HT = 0.3445, the within population diversity was higher (HS = 0.1755) than the between population diversity, which was DST = 0.1688. The proportion of total allelic diversity found among populations was GST = 0.4905. The within population diversity varied considerably from one population to another even in the same countries and ranged from 0.041 to 0.268 in three MOZ populations, 0.069 to 0.257 in three NAM populations,

Table 3. Genetic diversity parameters of populations from different countries.

Country of origin	Mean Nei's genetic diversity		% of polymorphic loci	% of rare bands at frequency of p<0.0
	Value	Rank		
MAD	0.2032	3	49.14	38
ZAM	0.2069	2	53.14	6
BOT	0.0000	8	-	0
KEN	0.1981	4	58.29	28
TZA	0.2126	1	69.71	25
ZIM	0.1816	5	45.14	3
NAM	0.1161	7	29,21	0
MOZ	0.1728	6	49.71	0

0.206 in 1 MAD population and 0.17 in 1 ZAM population. Table 3 shows the diversity parameters while Figures 6 and 7 show the level of polymorphism, country differences in diversity and rare bands as revealed by AFLP markers.

DISCUSSION

A study of the cluster analysis of relationships between countries reveal four major groups as shown in Figure 1 and the among country differentiation is significant at $p<0.05$. These major groups seem to be formed on the basis of a) the level of diversity according to Nei's indices b) the % of polymorphic loci and, c) the % of rare fragments at an arbitrary frequency of ≤ 0.02. A close analysis of the MAD populations shows that they are clearly distinct from the other groups. Compared to the other countries, the group had a high level of diversity, good level of % polymorphism, high % of rare fragments and the highest number of bands. Madagascar, where the populations originated, is an offshore island and the clear distinction from populations from the other countries may be attributed to environmental differences in addition to likely influences of the 'Equilibrium theory of Island biogeography' (MacArthur and Wilson, 1963; Brown and Lomolino, 1998) as reported by Kiambi et al. (2001). This is also consistent with the work on genetic variation in *Prunus Africana* showing that genetic distinctness and differentiation of populations may arise from geographic and ecological isolation (Dawson et al., 1999).

Genetic structure and diversity

Averaging of all loci, the total genetic diversity of HT = 0.3445 in the *O. longistaminata* populations is quite high and compares well with that of HT = 0.37 found in *Oryza glumaepatula* by Buso et al. (1998) using RAPDs. In their study of the South American populations of this species, they found much lower levels of within population diversity (0.081 and 0.089) but higher levels of between population diversity (0.28 and 0.29). This may be expected as they were working with an inbreeding species. In this study, the relatively high levels of mean diversity over all the loci and the higher within (0.1755) than between (0.1688) population diversity may be attributed to the mating system (Maki and Horie, 1999) since *O. longistaminata* is allogamous, has a partial self-incompatibility and cross pollination is favoured by reproductive organs especially anther and stigma (Chu et al., 1969). The average diversity over all the populations found by Parsons et al. (1999) in Bangladesh rice landraces was 0.200, which is lower than in the present study. This however may be expected since *O. longistaminata* is a wild species. In addition, more genetic variation is likely to be detected in a widely spreading taxon, particularly when the sampling is carried out over a wide geographical range (Maki and Horie, 1999), as was the case in this study.

Wright (1978) cited by Hartl (1987) suggested that an FST range of 0-0.05 indicates little differentiation, 0.05-0.15 moderate, and 0.15-0.25 large differentiation and above 0.25 indicates very large differentiation. In this study, the level of population differentiation was FST = 0.30 which is very large using the suggested parameters. Hamrick and Godt (1989) reported that selfing species are characterized by a relatively high HT value (0.33), a low HS value (0.15) and a high GST value (0.51). From the studies, *O. longistaminata* has a relatively high FST of 0.29, high HT value of 0.343 high GST value of 0.49 and a HS value of 0.175. With the exception of the HS value, all the other values are characteristic of selfing species although the difference in genetic diversity between selfing and outcrossing species at the species level are not statistically significant (Hamrick and Godt 1989). However, the higher within than between population diversity is characteristic of out-crossing species (Hamrick and Godt, 1989; Buso et al., 1998). Using RAPDs, Buso et al. (1998) working with *O. glumaepatula* found the following values: FST = 0.64 and

0.67, HT = 0.37, HS = 0.089, and 0.081, (DST = 0.29) and 0.28 GST = 0.63 and 0.65. They have reported that *O. glumaepatula*, which shares the same genome group of AA with *O. longistaminata*, is autogamous. In comparison, these figures reveal that the selfing tendencies are higher and more pronounced in *O. glumaepatula* than in *O. longistaminata*. Working on *Oryza barthii*, Bezançon (1993) reported a diversity index HT of 0.131, intra-population diversity HS of 0.025, inter-population diversity DST of 0.108 and a high coefficient of differentiation (GST = 0.82). These results are expected since the species is predominantly autogamous (Bezançon, 1993).

In many instances, species exhibit a spatial structure of genetic variation across their ecogeographic range. Different levels of genetic diversity among countries may be due to several factors including mating systems, rate of mutation, migration and dispersal mechanisms, biotic and abiotic selection intensities which are determined by location, climate and soil (Nevo et al.,1979; Frankel, 1984; Parsons et al., 1999; Kark et al., 1999.

In the estimates of total diversity, the proportion of diversity due to differences of populations within countries is higher than of among countries and there are distinct differences in diversity on the basis of geographic locations. For instance, TZA recorded the highest level of genetic diversity, the highest % of polymorphic loci and a high number of rare alleles in its populations. This may be due to environmental heterogeneity as reported by IUCN (1990) and Kiambi et al. (2001). In addition, the country generally seems to have environmental conditions and other factors that favour differentiation at both intra- and inter-specific diversity levels.

Working on distribution and ecotypic differentiation of wild *Oryza* species in Africa, Katayama (1987; 1990) found significant differences in variation of grain morphological characters in *O. longistaminata* populations collected from Kenya, Tanzania and Madagascar. Both inter and intra-population variation was highest in Tanzania materials, which agrees with the results showing that Tanzania has the highest diversity in the present study. The very low level of diversity between the NAM populations may be attributed to uniformity of the environmental variables as they were all collected along the floodplain of River Okavango. The river floods often and this may increase the gene flow by seed movement from one site to another along the floodplain. Buso et al. (1998) made the same observations when they found high similarities between populations of *Oryza glumaepatula* that had been collected from the same river basin.

Implications for conservation of wild rice genetic diversity

The results of AMOVA revealed significant differences ($p<0.05$) in genetic variation among populations within different countries of the region. Other genetic structure and diversity parameters including mean genetic diversity, band frequency and % of polymorphism have also indicated clear country differences in the structure of diversity.

The results also point to the need of developing sampling strategies that consider the population dynamics. For instance the NAM and MOZ populations have comparatively low between population diversity but show considerably higher within population diversity of 0.157 and 0.185 respectively, averaged from three populations. In these countries, it may be more cost effective to sample fewer populations but more individuals within those populations. In ZAM where the populations are high in diversity and show comparatively lower levels of within population diversity (0.175), it may be better to sample more populations but fewer individuals in those populations. These results are not conclusive and further studies are necessary for better understanding of the population dynamics, especially the mating system in *O. longistaminata* as this may influence the population structure and diversity.

In conclusion, the genetic parameters estimated from AFLPs data have indicated that there are high levels of genetic diversity in the wild populations of *O. longistaminata* studied and that this diversity is higher within than between populations. The hierarchical partitioning also revealed that most of this diversity is found between populations within countries than among countries. Regional collection and conservation strategies therefore need to consider country differences while national strategies should consider population differences within countries.

The study has clearly demonstrated the usefulness of AFLP in studying diversity in rice populations and its power of resolution in discriminating between populations and individuals within populations. It is clear that the study has also added significant knowledge and understanding of the population structure and genetic diversity of *O. longistaminata* in East and Southern Africa and its partitioning among countries, between and within populations.

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