Genetic Resources and Crop Evolution **43:** 203–210, 1996.

Application of random amplified polymorphic DNA to study genetic diversity in *Paspalum scrobiculatum* L. (Kodo millet, Poaceae)

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Received 13 September 1994; Accepted 9 May 1995

Key words: Paspalum, kodo millet, Poaceae, grasses, millets, RAPD, germplasm, genetic diversity

Summary

Genetic diversity and patterns of geographic variation among collections of *Paspalum scrobiculatum* (kodo millet) and *P. polystachyum* were studied using molecular markers generated through the random amplified polymorphic DNA (RAPD) method. A high level of polymorphism in RAPD markers was observed among the individual accessions, demonstrating the high genetic diversity of the crop. The markers obtained from the RAPD method were analyzed with the cluster analysis, principal coordinates and minimum spanning tree methods. Three major groups were resolved, one representing the African accessions, and two for the Indian accessions. The accessions of the north African kodo millet and *P. polystachyum* (considered conspecific with *P. scrobiculatum*) were quite distinct. The Australian kodo millet showed higher affinity to the African types. The study demonstrated that the RAPD technique can be applied to resolving degrees and patterns of genetic variation at the population and species levels, identifying cultivars, and defining gene pools of this crop.

Introduction

The genus *Paspalum* L. (Poaceae, tribe Paniceae) consists of about 330 species distributed over the tropical parts of the world in habitats ranging from savannahs to forests (Clayton & Renvoize, 1986). Whereas many species of *Paspalum* occur as weeds, some species are used for fodder, soil conservation or harvested in the wild.

Kodo millet (*Paspalum scrobiculatum* L.) is a cereal grown primarily in India where it was domesticated (Purseglove, 1972; de Wet et al., 1983). In Africa, the millet is either harvested in the wild or cultivated where it is known as bird's grass or black rice (Porteres, 1976). The wild form of *Paspalum scrobiculatum* is perennial, occurring throughout the tropics and subtropics of the Old World. In west Africa, it is a weed in rice fields and is harvested with the crop (de Wet et al., 1983). The cultivated form is grown as an annual, but some cultivars are capable of perennial growth (de Wet et al., 1983). The crop is hardy, drought tolerant, and can be grown under poor soil conditions ranging from gravelly to clay soils (de Wet et al., 1983; Purseglove, 1972). Kodo millet is thus highly suitable for sustainable agriculture in less arable regions such as the semi-arid areas. Millets are also becoming attractive crops in the developed countries for use in various food products, in malting, and as forage crops (Malleshi, 1989). At present, our knowledge of the amount and pattern of genetic variation in this crop is minimal.

Kodo millet is morphologically variable (de Wet et al., 1983; Dhagat & Singh, 1983). Three complexes are recognized on the basis of spikelet arrangement on the inflorescence; however, these variations do not support racial groups (de Wet et al., 1983). High genetic diversity among and within geographical regions has been documented (Dhagat & Singh, 1983). But, again, the "genetic diversity", which was equated with morphological variability, was not associated with geographical distribution. The lack of racial differentiation was attributed to multiple domestication of the crop along the range of distribution of its wild progenitor and to continue hybridization with the weedy and wild types.

Table 1. Species and accessions of *Paspalum* used in the study. Abbreviations were used in the figures and tables

Species	Abbreviation	Accession number	Area of origin
P. commersonii	Comm	Paltridge (KU-5584)	Japan, Kyushu Station
P. polystachyum	Poly	P.I. 224996	South Africa
P. scrobiculatum	Saf	P.I. 365511	South Africa
P. scrobiculatum	Tan	ILCA 861	Tanzania
P. scrobiculatum	Ken	ILCA 12838	North Eastern Kenya
P. scrobiculatum	Zai	P.I. 536032	Zaire
P. scrobiculatum	Nig	ILCA 13861	Niamey, Niger
P. scrobiculatum	Mor	P.I. 338662	Morocco
P. scrobiculatum	Aus	P.I. 420862	Australia
P. scrobiculatum	Ind1	P.I. 198332	India
P. scrobiculatum	Ind2	P.I. 463931	India, Gujarat State
P. scrobiculatum	Ind3	P.I. 198323	India, Kodra 18-4
P. scrobiculatum	Ind4	P.I. 198324	India, P.S. 1
P. scrobiculatum	Ind5	P.I. 198326	India, Kodra Cal. No. 138-1
P. scrobiculatum	Ind6	P.I. 463846	India, Andhra Pradesh State
P. scrobiculatum	Ind7	P.I. 463856	India, West Bengal State
P. scrobiculatum	Ind8	P.I. 463861	India, West Bengal State
P. scrobiculatum	Ind9	P.I. 463876	India, Bihar State

The recent development of the random amplified polymorphic DNA (RAPD) technique (Welsh & McClelland, 1990; Williams et al., 1990) has enhanced plant genetic and evolutionary studies. This molecular method is useful for estimating genetic variation at the population level and among closely related species (Williams et al., 1993). It has been utilized to study genetic variation in various crop plants, including cereals such as wheat (Devos & Gale, 1992; He et al., 1992; Joshi & Nguyen, 1993; Vierling & Nguyen, 1992), *Echinochloa* millets (Hilu, 1994) and *Panicum* millets (M'Ribu & Hilu, 1994). The technique has been found to resolve various levels of interspecific and intraspecific polymorphism which facilitates assessment of genetic relationships, definition of regional grouping, and identification of individual accessions or cultivars.

The objectives of this study were to determine the genetic diversity and observe patterns of variation in kodo millet over its geographic distribution and to examine its affinity to the allied *P. polystachyum*. The study also evaluated the utility of the RAPD technique for generating DNA markers for future studies at the population level in this crop.

Materials and methods

Plant material

Sixteen accessions of *Paspalum scrobiculatum* representing different geographic regions where the crop is cultivated, and one accession each of *P. polystachyum* R. Br. and *P. commersonii* Lam. were studied (Table 1). The accession of the latter two species included in this study were the only ones available. These two species are considered conspecific with *Paspalum scrobiculatum* based on morphological characteristics (Clayton, 1975). Plants were raised from seeds and grown in pots in the greenhouse. Leaf samples were harvested from five-week old, individual plants and stored at −80°C for later DNA extraction.

Template DNA preparation

Total cellular DNA was isolated from leaf material of a single individual plant following Hilu's modification (Hilu, 1995) of the method of Stewart and Via (Stewart & Via, 1993). Approximately 50–100 mg of tissue was placed in a 1.5 ml Eppendorf tube containing 300 μl of CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 0.7 m NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 8 and 0.1% mercaptoethanol). The tissue was macerated using a mechanical drill fitted with disposable plastic bits. Following maceration, an additional 300 μl of buffer was added, mixed thoroughly by gentle shaking and the suspension was incubated in a water bath at 60°C for 10 min. The suspension was mixed gently and spun lightly to pellet the plant debris. The aqueous portion was transferred to a new tube, mixed thoroughly with an equal volume (600 μ1) of chloroform and centrifuged at maximum speed for 1 min in a microcentrifuge. The aqueous phase was transferred to a new tube and DNA was precipitated by the addition of 400 μl of isopropyl alcohol and spinning for 5 min. The DNA was air-dried and dissolved in 50 μl TE buffer (Tris-EDTA, pH 8.0). Samples for polymerase chain reaction (PCR) amplification were diluted to approximately 5 ng/μl with de-ionized distilled water.

PCR amplification

Thirteen 10-base oligonucleotide primers (Operon Technologies, primers OPA 2, 4, 5, 6, 7, 10, 11, 13, 14, 16, 17, 18, 19) were randomly selected for PCR amplification to avoid bias toward those that reveal the most polymorphism (Clark & Lanigan, 1993). The RAPD procedure of Williams et al. (Williams et al., 1990) was used with modifications. Each reaction mixture (25 μl) contained 2.5 μl of 20× reaction buffer, 2 mM magnesium chloride, 10 mM each of dATP, dCTP, dGTP and dTTP, 0.047 μg of single PCR primer, 0.9 unit of Taq DNA polymerase (Promega), and approximately 15 ng of genomic DNA template. All reaction mixtures were prepared as master mixes for each primer to minimize measurement deviation that is more pronounced in small volume pipetting. The individual reaction mixture was overlaid with one drop of mineral oil. Amplification was carried out in a Perkin Elmer Cetus thermal cycler following Stewart & Via (1993): one initial denaturation cycle at 95°C for 5 min followed by 75 cycles of 94°C for 10 seconds, 36°C for 10 seconds and 72°C for 2 min. On completion of the cycles, samples were refrigerated at 4°C before electrophoresis.

The importance of the optimal concentrations of the components of the RAPD assay in the amplification is discussed by Williams et al. (1993). The concentrations of the components of the reaction mixture and the PCR amplification protocol are those routinely employed for our molecular studies of millets (Hilu, 1994; M'Ribu & Hilu, 1994). Different concentrations of DNA were tried to determine on the optimal amount required for successful amplification. To examine the reproductibility of the RAPD experiments, five representative genotypes were selected for amplification with six primers. Controls that contain all the components of the PCR amplification reactions except for the template DNA were included.

Amplification products were analyzed by electrophoresis on a 1.5% agarose gel. Approximately 20 μl of sample was loaded on the gel and run in Tris-acetate-EDTA (TAE) buffer at 100 V for 4.5–5 hours. The one-kilobase DNA ladder marker (Bethesda Laboratories) was used as a molecular standard. DNA was stained with ethidium bromide and photographed in UV light.

Data analysis

RAPDs behave as dominant markers (Clark & Lanigan, 1993), thus, they lend themselves to the bistate (present – absent) type of scoring. DNA bands that were present at lower intensities were scored as present. RAPD markers that were shared by all accessions were excluded from the data analysis since they are not informative (see Clark & Lanigan, 1993). In cases where template DNA (not individual DNA bands) of an accession was not well amplified, missing values were used in the scoring and incorporated in the matrix following the NYSYS-pc program directions (Rohlf, 1993). The raw data matrix was subjected to the Dice algorithm (Dice, 1945) to generate a matrix of similarity. The Dice coefficient is equivalent to equation 21 of Nei & Li (1979), as both calculate similarities from shared presence not abscence of DNA band. Similarity values obtained from the Dice alogorithm were utilized to group accessions via the Unweighted Pair Group Method (UPGMA). The cophenetic coefficients for the clusters were computed, and the correlation between these coefficients and the similarity matrix was computed with the normalized Mantel statistics z.

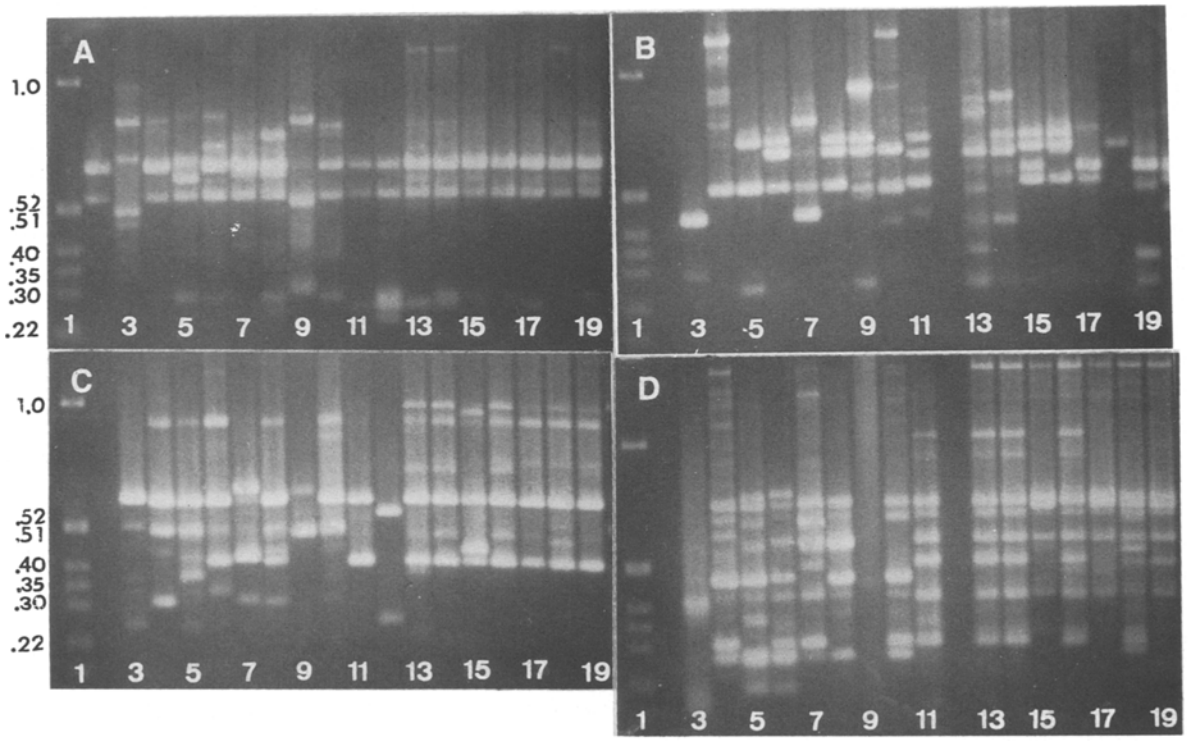

Fig. 1. Genomic DNA amplified by the RAPD method using primers 2 (1A), 5 (1B), 11 (1C), and 4 (1D). Lane 1 contains fragments of molecular weight markers labeled with the approximate size (kilobases). Lanes 2 to 19 represent amplified DNA from *Paspalum commersonii, P. polystachium* and *P. scrobiculatum* (kodo millet): Comm (2), Poly (3), Saf (4), Tan (5), Ken (6), Zai (7), Nig (8), Mor (9), Aus (10), Ind1 (11), Ind9 (12), Ind2 (13), Ind3 (14), Ind4 (15), Ind5 (16), Ind6 (17), Ind7 (18), Ind8 (19). The abbreviations of the accession names are taken from Table 1.

The Dice matrices of similarity were also used in a principal coordinate analysis (PCO) to resolve patterns of variation among the accessions. The principal coordinate has advantages over the principal component analysis for this type of data because the DNA bands represent qualitative data points (i.e. scored as present-absent) and the original matrix contains missing values (see Sneath & Sokal, 1973). The minimum spanning tree (MST) was also computed from the Dice similarity matrices and was projected onto the plot of the first three factors of the PCO. The MST will link nearest neighbour accessions (Rohlf, 1993). The NTSYS-PC computer program (Rohlf, 1993) was used to analyze the data.

Results and discussion

Variability in RAPD marker profiles was observed among the accessions (Fig. 1). Two accessions representing *P. commersonii* and kodo millet accession Ind9 (Table 1) were excluded because of poor DNA amplification with various primers, affecting large sectors of the lanes, but not individual bands (Fig. 1, lanes 2 and 12). This difficulty of amplification from these two templets has resulted in a large number of DNA fragments scored as missing data, making the inclusion of these two accessions in the analysis impractical. Primer 7 amplified one DNA band of the same molecular weight all across the accessions examined; consequently, it was excluded from the analysis since it was not informative. The 12 remaining primers generated a total of 285 markers. The markers ranged in size from about 200 to 1000 bp (base pair). Approximately 98% of the markers were polymorphic. Primers varied greatly for the ability to resolve variability among the accessions. Whereas some primers (e.g. OPA-4 and 11, Fig. 1C,D) generated several markers and were able to show high genetic diversity, others (e.g. OPA-2, Fig. 1A) generated fewer markers and showed little variability.

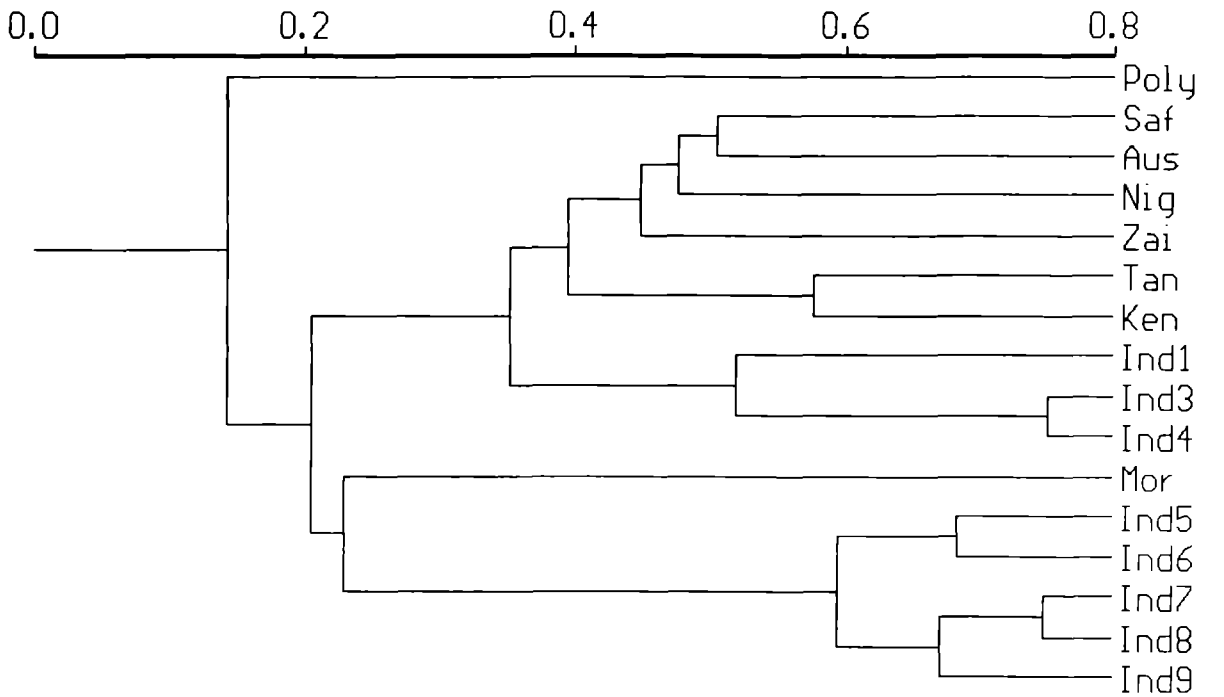

Fig. 2. Grouping of the kodo millet (*Paspalum scrobiculatum*) and *P. polystachyum* accession besad on the analysis of the RAPD data with the Dice similarity coefficient and the UPGMA method. Abbreviations of the names follow Table 1.

Similarity between individual accessions within the domesticated species varied from 21% to 75%, indicating a very high degree of genetic diversity (Fig. 2). This molecular information concurs with the reported high morphological diversity in kodo millet (de Wet et al., 1983; Dhagat & Singh, 1983).

The accessions are classified into three major groups by the markers. The Indian accessions segregated in two, well defined clusters named Indian groups 1 and 2. Accessions of African origin and the Australian collection formed the third group. The accession from Morocco grouped loosely (at 0.2 similarity) with one of the Indian. *Paspalum polystachyum* was the least similar accession, grouping with the *P. scrobiculatum* individuals at 14% similarity.

The Indian accessions formed two clusters referred to here as Indian group 1 (accessions Ind 1, 3, 4) and Indian group 2 (accessions Ind 5, 6, 7, 8, 9). The two clusters formed by the Indian accession were very distinct (21% similarity coefficient), with group 1 clustering with the African-Australian accessions. The distinctness of the Indian groups was quite apparent from the general RAPD markers pattern. Variability in RAPD markers was generally low among the Indian accessions compared with the others (Fig. 1A,C,D). Within Indian group 2, accessions from West Bengal and Bihar, two neighboring states in northeastern India, showed close affinities. Grouped with these two was an accession from Andra Pradesh which is also a western state, but it is located further south. The collections from Kodra, however, were spread among the two Indian groups. Therefore, it is difficult to conclude that a clear cut geographic pattern is apparent. The RAPD-based association observed among the Indian accessions did not correspond to the plant height and leaf length information provided by the U.S. Department of Agriculture Southern Regional Plant Introduction Station (unpublished information). This is not surprising, since morphological studies based on vegetative and reproductive traits underscored the lack of racial differentiation (de Wet et al., 1983; Dhagat & Singh, 1983).

The accessions from Africa, although appearing in one cluster, were quite diverse as reflected by the lower affinities among them compared with those observed within each Indian subgroup (Fig. 1C,D, Fig. 2). Among the African Kodo millet, the accessions from the neighboring countries Kenya and Tanzania showed the highest affinities, grouping at 60% similarity. The higher diversity in the African collection could imply

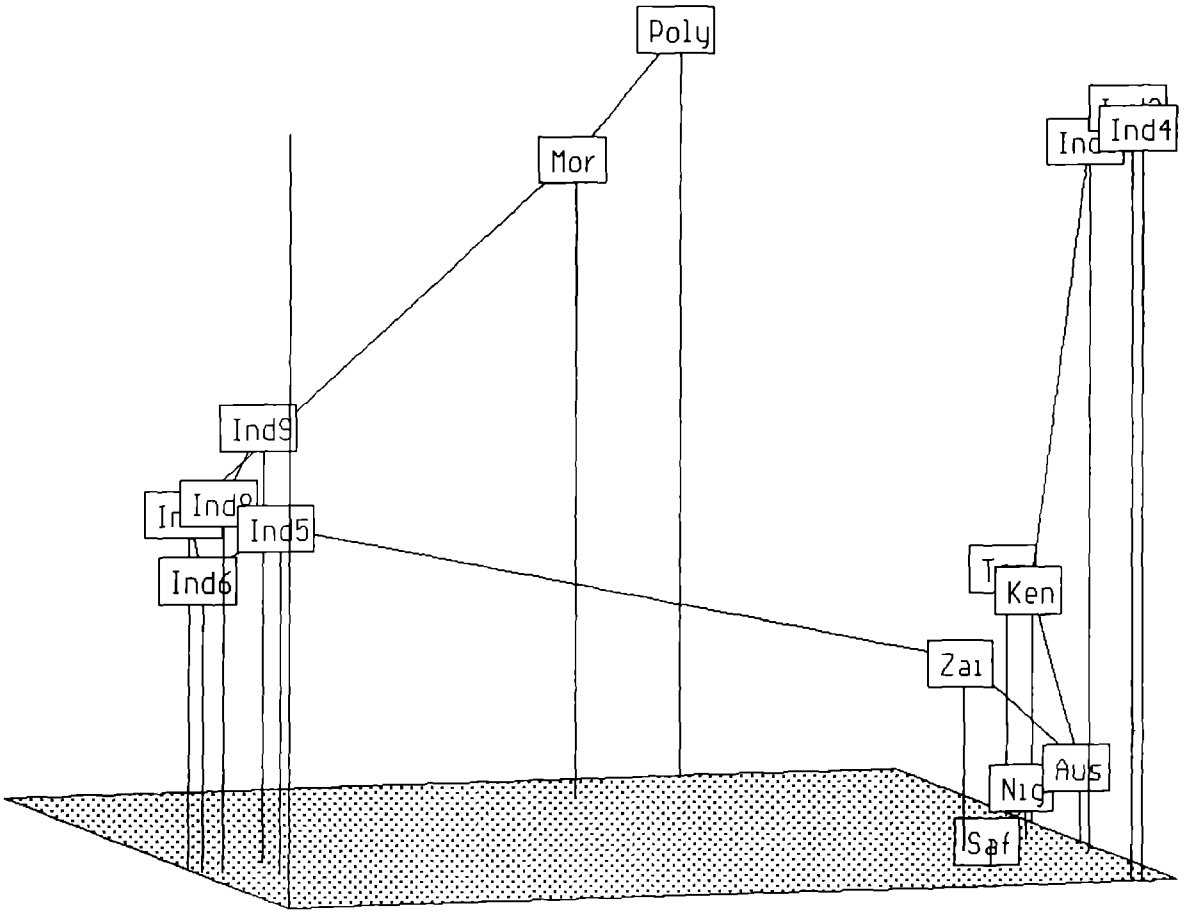

Fig. 3. A three dimensional plot of the principal coordinate analysis with the minimum spanning tree superimposed on the plot. Abbreviations of the names follow Table 1.

multiple introductions of kodo millet from Indian types of group 1.

The principal coordinate (PCO) and minimum spanning tree (MST) analyses provided additional information on the pattern of variation observed in kodo millet and its affinities to *P. polystachyum*. The first three eigenvectors accounted for 50% of the variation. In the three-dimension plot based on the first three factors, the three major groups representing the two Indian and the African clusters were quite distinct (Fig. 3). Indian group 1 appeared closer to the African collections in the first two factors. The Moroccan collection and the *P. polystachyum* genotypes were adjacent to each other and show similarity on the three factors. Superimposing the MST onto the PCO provided additional useful information (Fig. 3). The three major groups revealed by the cluster analysis were confirmed as separate entities because the MST connected all genotypes within each group first before joining a nearest neighbor from another group. This MST information also indicate lack of distortion in the pattern resolved in the PCO plot. Interestingly, the two Indian groups were not connected directly to each other, reflecting the high divergence between them. The African accessions were linked to the two Indian groups (Fig. 3), implying possible multiple introduction of kodo millet to Africa as ancient trading is well documented between these two regions (Hornell, 1941; Brentjes, 1967).

The Australian accession was connected to four of the five African ones which implies general affinities to the African kodo millets. The Kenyan genotype to which the Australian kodo millet was connected was linked directly to the Indian group 2 millets, implying that the Australian type is only one step removed from the Indian collections. The PCA and MST (Fig. 3) fur-

ther confirmed the cluster analysis results (Fig. 2) in showing the distinctness of the accession of the tropical African species *P. polystachyum* and the Moroccan accession from the rest of the African types (Fig. 3). The *P. polystachyum* accession was connected directly with the Moroccan accession which, in turn, was connected to the Indian group 2 but not with the other African kodo millets. The MST did not show an association between the North African genotype from Morocco and the subSaharan kodo millets, reflecting genetic divergence due probably to a distinct origin. Clayton (Clayton, 1975) indicated that *P. scrobiculatum* and *P. polystachyum* are morphologically very similar and consequently placed them in the same species. The accession examined of *P. polystachyum* does not quite reflect that assertion; however, only one accession was available for this molecular study and the extent of diversity in this wild species must be evaluated before a reliable conclusion can be made.

Unlike the morphological studies (de Wet et al., 1983; Dhagat & Singh, 1983), systematic analysis of the RAPD markers in kodo millet effectively separated the Indian from the African cultivars and resolved some geographic trends within each region. The resolution provided by the RAPD method for studying patterns of genetic variation in germplasm resources was also demonstrated by our previous studies of sawa and barnyard millets (*Echinochloa* species), finger millet (*Eleusine* species), Hilu 1995, and proso millet (*Panicum miliaceum* L.) (Hilu, 1994; M'Ribu & Hilu, 1994). The high polymorphism revealed among individual accessions of kodo millet illustrates that the RAPD technique is an effective descriptor of intraspecific variation in the crop. The technique can resolve genetic variation and identify cultivars in the breeding programs of the crop. Grouping germplasm into geographical entities and elucidating affinities among these groups can define gene pools and determine gene flow among populations.

Acknowledgements

We thank the U.S. Department of Agriculture Southern Regional Plant Introduction Station for supplying the seed material. This research was supported under grant No. DHR-5600-G-00-1073-00, Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development.

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