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Detection of interspecific and intraspecific variation in *Panicum* millets through random amplified polymorphic DNA

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Abstract The potential use of random amplified polymorphic DNA (RAPD) was evaluated as a source of genetic markers for studying variation among four species of *Panicum* and within the crop species *P. miliaceum* and *P. sumatrense*. Polymorphism in RAPD markers was observed across and within species. The four species were distinct in RAPD patterns and were separated at low correlation values even with small samples involving single genotypes per species. Accessions of *P. miliaceum* were grouped according to geographical regions of origin. The study demonstrated that unlike isozyme and protein electrophoresis patterns, RAPD markers can be applied to studying genetic diversity, defining gene pools, and identifying cultivars for this group of millets.

Key words *Panicum* · Millet · RAPD · PCR · Genetic diversity

Introduction

Small millets are important cereals for the arid and semi-arid regions of the world. These crops provide food and fodder and have a high potential for the development of sustainable agricultural systems in these areas. Three of the small millets belong to the genus *Panicum*: proso millet (*Panicum miliaceum* L.), sama (little or slender) millet (*Panicum sumatrense* Roth. ex Roem. et Schult., synonym: *Panicum milliare* Lamk.), and sauwi millet (*Panicum sonorum* Beal). In addition to the crop species, the weedy species witch grass (*P. capillare* L.) has been harvested for food from the wild but does not appear to have been developed as a crop (Doebley 1984; Harlan 1992). *Panicum miliaceum* and *P. sumatrense* are tetraploid (2n = 36) species of Old World origin, wheras *P. capillare* and possibly *P. sonorum* are diploid (2n = 18) species of the New World (Purseglove 1972; Tsvelev 1976; Hiremath et al. 1990). Our knowledge of the genetic diversity and gene pool structure of small millets is minimal. Consequently, to facilitate the development of these cereals, studies need to be carried out to determine their genetic diversity and to define their gene pools for use in selection and breeding.

Proso millet (*P. miliaceum*) is a temperate crop cultivated in central Europe, USSR, China, India, and the Middle East (de Wet 1989; Doggett 1989; Harlan 1992). It is cultivated to a small extent in North America where it also occurs as a weed escaping cultivation (Bough et al. 1986; Colosi et al. 1988; Wilson and Westra 1985). The specific area of domestication of proso millet is not known; however, central Asia, China, or central Europe have been proposed as possible regions (de Wet 1989; Doggett 1989; Harlan 1992). While proso displays a high degree of morphological variability across its range of distribution, this variation does not fall into geographic or taxonomic grouping, and attempts to use isozymes and protein electrophoresis for intraspecific grouping or classification have not been successful (Warwick 1990; L. Oestry, unpublished data).

Sama millet (*P. sumatrense*) is cultivated in the Indian subcontinent and southeastern Asia (de Wet et al. 1983; Hiremath et al. 1990; Hiremath et al. 1991). The crop was domesticated in India (de Wet 1989; de Wet et al. 1983; Harlan 1992) from the wild and weedy grass *P. psilopodium* (de Wet 1989; Hiremath et al. 1990, 1991). Cultivars of sama millet have generally been developed in response to natural and human selection with little conventional breeding (Reddy et al. 1984). De Wet et al. (1983) recognized two morphological races in sama millet on the basis of inflorescence and plant height characteristics.

Sauwi millet (*P. sonorum*) is an ancient minor cereal native to arid southwestern North America. The crop

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was domesticated in and has been endemic to the Sonoran region of northwestern Mexico where it once counted among the major food plants (Doebley 1984; Nabhan and de Wet 1984; Nabhan 1985; Harlan 1992).

The development of molecular techniques has greatly enhanced plant genetic studies. Genetic variation is commonly detected through polymorphic molecular markers generated by isozymes, protein eletrophoresis patterns, and restriction enzymes. More recently, the random amplified polymorphic DNA (RAPD) technique has emerged as another means of estimating genetic variation at the population and species levels (Welsh and McClelland 1990; Williams et al. 1990). In the RAPD technique, genomic DNA is amplified by the polymerase chain reaction (PCR) using arbitrary primers. This technique has been utilized to study genetic variation in various organisms, including crop plants such as tomato (Klevin-Lankhorst et al. 1991), wheat (Devos and Gale 1992; He et al. 1992; Vierling and Nguyen 1992), cabbage, cauliflower, broccoli, and other cruciferous crops (Demeke et al. 1992), groundnut (Halward et al. 1992; Lanham et al. 1992), potato (Quiros et al. 1993; Singsit and Ozias-Akins 1993; Xu et al. 1993), and *Echinochloa* millets (Hilu 1994).

The objectives of this study were to determine the genetic differences among *Panicum* millets, to observe patterns of variation within the crops, and to evaluate the applicability of the RAPD technique in generating DNA markers for genetic and breeding studies of these millets.

Materials and methods

Plant materials

Thirteen accessions of *Panicum miliaceum*, 3 of *P. sumatrense* and 1 each of *P. sonorum* and *P. capillare* were studied (Table 1). Unavailability of seed material for *P. sumatrense* and *P. sonorum* limited the sample size. *Panicum capillare*, a cosmopolitan weed of North American origin (Baskin and Baskin 1985), used as a reference species. Plants were raised from seeds and grown in pots in the greenhouse. Leaf samples were harvested from 7-weak-old individual plants and preserved in the freezer (−70 °C) for DNA extraction.

Template DNA preparation

Total cellular DNA was isolated from leaf material following the method of Saghai-Maroof et al. (1984) as modified by Hilu (1994). Approximately 1–2 g of tissue was ground in liquid nitrogen and suspended in 15 ml 2% CTAB extraction buffer (2% hexadecyltrimethylammonium bormide, 0.7 *M* NaCl, 10 m*M* EDTA, 50 m*M* TRIS-HCl, pH 8, and 0.1% mercaptoethanol). The mixture was incubated in a waterbath at 60 °C for 45 min with gentle shaking at 10 min intervals. The suspension was allowed to settle in the final 10 min, and the supernatant was transferred to new tubes. An additional 5 ml of buffer was added to the ground material, and the mixture was incubated for 20 min. The two portions were then combined, mixed thoroughly with 15 ml chloroform and centrifuged at 6000 *g*. The DNA was precipitated from the aqueous phase with 14 ml isopropyl alcohol, spun at 6000 *g* for 10 min, air-dried, and dissolved in TE buffer (TRIS-EDTA, pH 8.0). The 2% concentration of CTAB extraction buffer and the two-step extraction procedure has been found to increase the quantity of DNA extracted (Hilu 1994). The concentration of DNA in the samples was determined with a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments). Samples for PCR were diluted to approximately 5 ng/μl with deionized distilled water.

Table 1 Sources of plant materials used in the study. Accessions with PI numbers were provided by the US Department of Agriculture, and the ILCA accession was provided by the International Livestock Center for Africa, Addis Ababa, Ethiopia

Species	Code number	Accession	Origin
P. miliaceum	M48	PI 222811	Iran
	M49	PI 223391	Iran
	M51	PI 250979	Yugoslavia
	M53	PI 269958	Pakistan
	M55	PI 289327	Hungary
	M57	PI 290726	United Kingdom
	M62	PI 408805	China
	M64	PI 463090	India
	M65	PI 463253	India
	M69	PI 476400	USSR
	M70	PI 476401	USSR
	M74	PI 517017	Morocco
	M75	PI 531401	Hungary
P. sumatrense	Su 14	PI 463534	India
	Su 15	PI 463652	India
	Su 22	PI 463670	India
P. sonorum	So 91	ILCA 12558	Unknown
P. capillare	Ca 20	PI 220025	Afghanistan

PCR amplification

Eleven 10-base oligonucleotide primers (Operon Technologies, Table 2) were randomly selected for RAPD PCR amplification following the procedure of Williams et al. (1990) with some modifications. Each reaction mixture (25 μl) contained 2.5 μl of 20 × reaction buffer, 2 m*M* magnesium chloride, 10 m*M* each of dATP, dCTP, dGTP and dTTP, 0.047 μg single PCR primer, 0.9 units Taq DNA polymerase (Promega), and approximately 15 ng genomic DNA template. The mixture was overlaid with two drops of mineral oil. Amplification was carried out following the procedure of Stewart and Via (1993) in a Perkin Elmer Cetus thermal cycler as follows: 1 initial denaturation cycle at 95 °C for 5 min followed by 75 cycles of 94 °C for 10 s, 36 °C for 10 s, and 72 °C for for 2 min. On completion of the cycles the samples were refrigerated at 4 °C before analysis.

PCR amplification products were analyzed by electrophoresis on a gel containing 0.7% agarose and 1.0% Synergel. The whole reaction sample (25 μl) was loaded on the gel and run in TAE (TRIS-acetate-EDTA) buffer at 120 V for approximately 5 h. A 1-kb DNA ladder marker (Bethesda Laboratories) was used as a molecular standard. DNA was stained with ethidium bromide and photographed in UV light.

Data analysis

Genotypes were scored for the presence or absence of RAPD markers. Markers of a low intensity were scored as being present. The four species were compared in three different ways in order to investigate the influence of varying the number of genotypes of *P. miliaceum* and *P. sumatrense* on the determination of differences at the species level. One accession each of *P. sonorum* and *P. capillare* was used in all three sets of analysis. The data sets differed in the number of genotypes of *P. miliaceum* (13, 3, or 1) and *P. sumatrense* (3, 3, or 1). In all cases, only

those markers generated by the genotypes in each particular set of accessions were considered. In addition to these analyses, intraspecific comparisons were made among the 13 genotypes of *P. miliaceum* and among the 3 of *P. sumatrense* using only the markers generated by those genotypes.

RAPD data were analyzed with the Dice algorithms (Dice 1945) to generate matrices of similarity using the NTSYS-PC computer program (Rohlf 1988). The Dice algorithm is identical to Nei and Li's measure (Nei and Li 1979, Eq. 21); both calculate the degree of affinities on the basis of shared attributes as a proportion of the total. The algorithms consider only shared presence but not shared absence of a DNA band (attribute) as a measure of similarity. Similarity values from the matrices were utilized to group genotypes via the unweighted pair group method (UPGMA) using the NTSYS-PC computer program (Rohlf 1988).

Results and discussion

A total of 294 scoreable RAPD markers varying in size between 170 and 3790 base pairs (bp) were generated with the 11 primers (Table 2). Primers varied in the information they generated, with some resolving high genetic diversity wheras others showed little or no variability. Some DNA markers were shared among the four species, wheras others were unique to species or genotypes (Fig. 1). Two markers generated by primer OPA-02 and OPA-13 were observed across accessions of all species. These 2 invariable markers may have been amplified from highly conserved regions of the genome, and they may represent conserved DNA sequences in the genus.

Variation among species

The four species were distinct in their RAPD marker profiles (Fig. 1). The total and unique number of markers varied with the species and number of accessions analyzed (Table 3). On the basis of all 18 accessions, approximately 32%, 28%, 20% and 22% of the RAPD markers were unique for *P. miliaceum*, *P. sumatrense*, *P. sonorum* and *P. capillare*, respectively. The mean percentage of shared markers between two species ranged from 21 to 40.

Table 2 Nucleotide sequence of the 11 oligonucleotide primers and the total number of RAPD markers they generated in the 18 genotypes of *Panicum* species

Primer	Nucleotide sequence (5′ – 3′)	Number of markers
OPA-02	TGCCGAGCTG	27
OPA-05	AGGGGTCTTG	29
OPA-06	GGTCCCTGAC	34
OPA-07	GAAACGGGTG	21
OPA-08	GTGACGTAGG	22
OPA-11	CAATCGCCGT	31
OPA-12	TCGGCGATAG	26
OPA-13	CAGCACCCAC	25
OPA-15	TTCCGAACCC	17
OPA-17	GACCGCTTGT	38
OPA-20	GTTGCGATCC	24

The total number of markers and proportions of unique and invariable markers are valuable parameters in determining intraspecific variability and genetic relationships among species. Although specific values of these parameters varied with number of genotypes per species (Table 3), it appears that a sufficient number of markers can be generated by a few selected primers to enable comparison of species with small samples. The grouping pattern for the four species was identical for the three data sets when 11 primers were used (Fig. 2). The degree of correlation among the species was also identical for the data sets with 18 and 8 genotypes. Reducing the number of genotypes to one per species only slightly altered the correlation values but not the

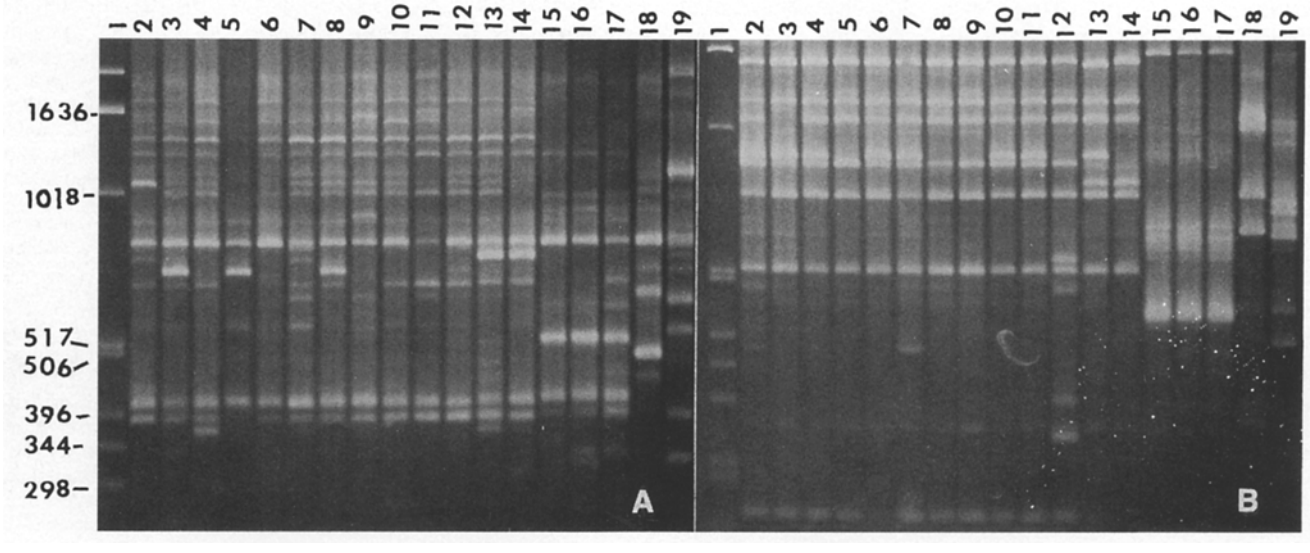


Fig. 1A, B Gel electrophoresis patterns of RAPD markers generated by primers OPA-02 (**A**) and OPA-11 (**B**) for 18 genotypes of the four *Panicum* species. *Lane 1* 1-kb ladder marker, *2–14 P. miliaceum, 15–17 P. sumatrense, 18 P. capillare, 19 P. sonorum.* In **1B** the double band of the ladder marker (third from the top in lane 1) corresponds to the 506- and 517-bp fragments

Table 3 Number of RAPD markers observed for the four species in three sets of experiments where 18 genotypes (A), 8 genotypes (B), and 4 genotypes (C) were used

Species	Number of genotypes analyzed			Total number of markers for species			Number of markers unique to species		
	A	B	C	A	B	C	A	B	C
P. miliaceum	13	3	1	199	157	115	63	56	39
P. sumatrense	3	3	1	108	108	89	30	39	35
P. sonorum	1	1	1	101	101	101	20	24	27
P. capillare	1	1	1	102	102	102	22	26	33

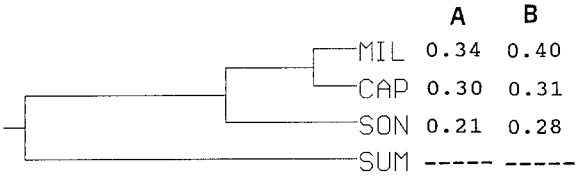


Fig. 2 Association pattern among *Panicum miliaceum* (*MIL*), *P. sumatrense* (*SUM*), *P. capillare* (*CAP*), and *P. sonorum* (*SON*) based on the Dice similarity matrices. *Numbers on the right* represent UPGMA similarity values among species. *A* Association based on 8 or 18 genotypes, *B* association based on 4 genotypes

pattern (Fig. 2). This implies that the RAPD method can be used to discriminate among species even when only a few accessions are available.

The four species separated at low correlation values in the three data sets (Fig. 2), suggesting that they are not closely related. *Panicum miliaceum* and *P. sumatrense* are native to Asia, whereas *P.* capillare and *P. sonorum* are native to the Americas. The later two species have been considered to be morphologically related (Nabhan and de Wet 1984), although this assumed relationship is not reflected by the RAPD information. However, a definitive conclusion regarding species' evolutionary relationships may require a larger sample. The RAPD data do not provide evidence for a closer relation between the two Old World crop species *P. sumatrense* and *P. miliaceum*; the former assumed a distinct position in the phenogram (Fig. 2). The wild ancestor of *P. sumatrense* is believed to be *P. psilopodium* (de Wet et al. 1983; Hiremath et al. 1990, 1991), whereas the progenitor of *P. miliaceum* is not known (Harlan 1992). This lack of close association among the species suggests that they were domesticated from distantly related species and that they may not have significant interspecific cross breeding.

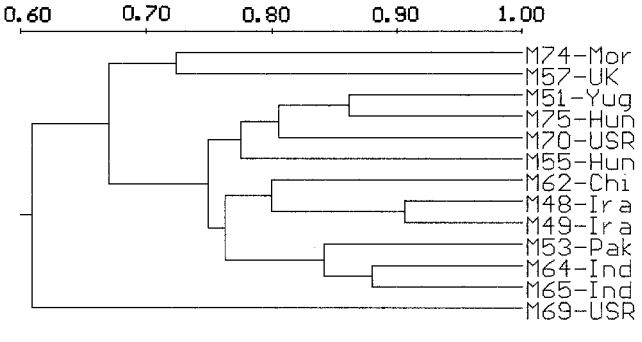
Variation within proso and sama millets

A high degree of variability in RAPD markers was observed in proso millet with approximately 73% of the 199 markers being polymorphic. The correlation between individual accessions varied from 52% to 91%, which indicates high genetic polymorphism. Proso millet is morphologically variable; however, the variation is continuous and no discrete grouping can be ascertained (L. Ostrey, unpublished data). Although subspecies, races, or biotypes have been proposed (Bough et al. 1986; Colosi et al. 1988; de Wet 1989; Harlan 1992; Warwick 1990), it has been observed that the races may not have eco-geogaphic unity (de Wet 1989) and that the weedy and wild forms are often indistinguishable from the crop (Harlan 1992). Warwick (1990) observed a very low level of allozyme polymorphism in *P. miliaceum*. Similarly, L. Oestry (personal communication) did not observe variation in isozyme, chromosome, or protein profiles.

The polymorphism observed in RAPD markers among cultivars of proso millet in the present study demonstrates the effectiveness of this method in determining intraspecific variation. The RAPD method thus provides an effective tool for studying genetic diversity within the crop. In addition, 17 unique and invariable markers were observed in this millet, and these can potentially be used as molecular fingerprints to identify the species.

The RAPD data grouped proso cultivars according to their geographical origin (Fig. 3). Cultivars from each country grouped together, and those representing geographic regions showed high affinity. The genotypes from Morocco and the United Kingdom appeared as a loose group. Three other groups representing Europe, China-Iran, and Pakistan-India were also resolved. The Asian genotypes showed more genetic affinities toward each other than to the European ones, and the genotypes from India and Pakistan were closely related. The USSR cultivar (M69, Fig. 3) appeared in an isolated position, which is probably due to a high number of missing data points. This information is valuable for defining gene pools and developing breeding programs for the crop. The observed association of cultivars from contiguous regions may be a result of similar natural or human selection within the regions or due to seed movement and gene flow.

Fig. 3 UPGMA association on 13 genotypes of *P. miliaceum* based on the Dice similarity matrix. The scale represents UPGMA similarity values. Sources of the accessions are indicated. *Chi* China, *Hun* Hungary, *Ind* India, *Ira* Iran, *Mor* Morocco, *Pak* Pakistan, *UK* United Kingdom, *USR* USSR, *Yug* Yugoslavia



Diversity in RAPD markers for the 3 sama millet accessions was also evident, with one-half of the 108 RAPD markers being variables. The correlation between genotypes ranged from 50% to 74%. This degree of variability obtained from only 3 genotypes is comparable to that of proso millet, a widely distributed crop. Sama millet is grown primarily in India where the 3 genotypes originated. It is morphologically variable (de Wet et al. 1983; Reddy et al. 1984), which is reflected by the high genetic diversity resolved by the RAPD analysis.

The RAPD method appears to be an effective approach in resolving genetic variations in *Panicum* millets, fingerprinting species and cultivars, and grouping germplasm into geographical races. This is in contrast with morphological and other molecular methods that did not show this depth of resolution. The potential of this approach in enhancing the improvement of these millets via selection and breeding needs to be further explored and exploited.

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