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Mitochondrial DNA variation in finger millet (*Eleusine coracana* L. Gaertn)

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Summary

This study was conducted to classify 26 lines of finger millet from Africa and India into cytotype groups based on the Southern blot hybridization patterns obtained with maize and sorghum mitochondrial cloned gene probes. Five restriction endonuclease enzymes were used for single digestions on total cellular DNA, giving a total of 20 enzyme/probe combinations. There was a low level of polymorphism, with identical RFLP banding patterns in 23 of the 26 lines. However, mtDNA clone *atp9* hybridized to a 3.6 Kb *Xba*1 fragment in ecotype SDFM-1143 from Malawi; but did not hybridize to a 3.0 Kb fragment present in all other ecotypes. Two Zimbabwean lines, SDFM 63 and SDFM 77, had an extra, low intensity 6.5 Kb *Xba*1 fragment hybridized by mtDNA clone *cox1*. This data enabled classification of the lines into 3 cytotype groups.

Introduction

Finger millet (*Eleusine coracana* L. Gaertn) is an important food crop in Africa and Asia. The probable area of origin of finger millet is the east African highlands stretching from Ethiopia to Uganda (Hilu & de Wet, 1976), while India (Asia) is a secondary center of diversity (Hilu et al., 1979). Finger millet is a highly self fertilized allotetraploid (2n = 36 chromosomes) derived from the wild tetraploid progenitor *Eleusine coracana* subsp. *africana*. One of the genome donors is believed to be the diploid *Eleusine indica* (Hilu, 1988). According to Hilu & de Wet (1976) finger millet has radiated in several directions in both Africa and Asia resulting in new races.

Cytoplasmic genes encoded by the chloroplast or mitochondrial DNA (cpDNA and mtDNA) can control important agronomic traits such as male sterility and resistance to diseases and herbicides. The discovery of possible deleterious organelle encoded genes has prompted crop breeders to identify and utilize the cytoplasmic diversity that is available among germplasm resources (Weissinger & Albertson, 1984). Restriction fragment length polymorphism (RFLP) has been used to characterize cytoplasmic genetic variation in many crop species (Kemble et al., 1980, 1983; Levings & Pring, 1977; Borck & Walbot, 1982; McNay et al., 1983; Gengenbach & Connelly, 1981; Chowdhury & Smith, 1988; Smith & Chowdhury, 1989; Khairallah et al., 1991). RFLP analysis has been effective in identifying mtDNA and cpDNA genotypes among lines which do not differ by easy to score, cytoplasmically controlled phenotypes such as chlorophyll deficiencies or male-sterility.

Previous RFLP studies on finger millet have suggested that a low level of cytoplasmic variation exists in this species. Using ten enzymes to generate restriction fragments, Hilu (1988) found no cpDNA variation among three domesticated finger millet accessions and three accessions of the wild progenitor (*E. coracana* sbspp. *africana*). In a separate study, Hilu (1992) also found no variation within the nuclear encoded ribosomal DNA (rDNA) spacer region in 26 finger millet accessions. Since the chloroplast genome is more conserved than the mitochondrial genome (Palmer, 1985), a molecular analysis of the latter is more likely to show RFLP variation. This has been demonstrated in maize and pearl millet. MtDNA variation in finger millet had not yet been studied to date. Sampling a wider array of accessions representing the entire ecogeographic dis-

tribution of this crop species should increase the probability of detecting cytoplasmic variation. Our objective in this study was to classify 26 lines of finger millet from different ecogeographic regions of Africa and Asia into possible cytotype groups based on Southern blot hybridization patterns with maize and sorghum mitochondrial gene probes.

Materials and methods

Plant materials and DNA isolation

Twenty-six morphologically diverse lines of finger millet covering the full geographic distribution of the crop in Asia and Africa were used in this study (Table 1). The seed of these varieties was kindly provided by Dr. S.C. Gupta of SADCC/ICRISAT in Zimbabwe (lines 1–17) and Prof. D.J. Andrews of the University of Nebraska-Lincoln (UNL) (lines 18–26). In addition, a sorghum line (N108 with the B cytoplasm, kindly provided by Dr. Jeff Pedersen, USDA-ARS, UNL) and a maize line (a cold tolerant inbred line selected from UNL population B, kindly provided by Dr. Blaine Johnson, Agronomy Department, UNL) were used to evaluate the effectiveness of the sorghum and maize mitochondrial clones in hybridizing mtDNA-specific restriction fragments. Five seedlings of each line were grown in pots in the greenhouse in January 1991. Six-week-old seedling shoots were cut and pooled together for each entry. The tissue was then lyophilized for 24 hours and stored at - 20° C. Total DNA was extracted from 300–500 mg of lyophilized tissue of each of the 26 varieties using a procedure adapted from that of Saghai-Maroof et al. (1984).

Restriction endonuclease digestion and electrophoresis of total DNA

Total DNA (approx. 10 μg) of each sample was digested singly with the following five endonuclease restriction enzymes: *Eco*RI, *Bam*HI, *Hind*III, *Eco*RV, and *Xba*1; according to the manifacturer recommendations (Boehringer-Mannheim). Completely digested total DNA was then dissolved in 25 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl of pH 7.4, 1 mM EDTA of pH 8.0), and loaded onto horizontal 0.8% agarose gels and electrophoresed at 60 V for 12 hours in 0.5 X TRIS-borate (TBE) buffer (0.045 M Tris-borate, 0.001 M EDTA). A lane of *Hind*III digested Lambda DNA was also included to provide markers for restriction fragment size estimation. Gels were stained with ethidium bromide and photographed on a UV transilluminator.

Southern transfers and probe labelling

DNA was transferred from the gels onto nylon membranes (Southern, 1975) with a neutral transfer protocol, as recommended by the manufacturer of the membranes (Boehringer-Mannheim). The membranes were then baked in an oven at 60° C for 3 hours.

Four mitochondrial gene clones were used for hybridizations and were kindly provided by Dr. S.C. Levings of North Carolina State University. Turf2B is a 9 kilobase pair (Kb) fragment which includes the T-*urf13* gene and *orf25* from cms-T maize mitochondria. *Cox1* is a clone encoding the cytochrome c oxidase subunit I, from *Zea diploperensis*. The clone *atp9* is a 2.2 Kb fragment which contains a gene for the ATPase subunit 9, from sorghum mitochondria; while 18S-5S is a 6 Kb rRNA clone also from *Zea diploperensis*. Insert DNA from plasmid minipreps (Birnboim & Doley, 1979) was labelled by the random primer method (Feinberg & Vogelsten, 1983) with digoxigenin-dUTP for use as probes in the immunochemiluminescent detection of filter-bound DNA (Boehringer-Mannheim). Before hybridization, the labelled probe DNA was denatured in boiling water for 10 minutes and cooled rapidly on ice for 3 minutes. About 20 μl of TE buffer with approximately 50 ng of labelled probe DNA in 20–25 ml of hybridization solution was used for hybridization for each filter.

Hybridization and illuminography onto X-ray film

The nylon membranes were first placed in sealed plastic bags with 20-25 ml hybridization solution (5X SSC (3 M NaCl, 1 M sodium citrate), 0.1% N-laurylsarcosine, 0.2% SDS (w/v) (sodium dodecyl sulfate) and 1% blocking agent). Bubbles were removed and the membranes prehybridized in a 68° C water bath for 12–20 hours. The hybridization solution was then replaced by 2 ml of fresh hybridization solution with the denatured probe. The bags were resealed and incubated at 68° C for 24 hours.

The membranes were removed from the bag and washed twice at room temperature with at least 50 ml of 2X SSC, 0.1% SDS per 100 cm^2 membrane. This was followed by washing twice for 15 minutes at 68° C with 0.1 X SSC, 0.1% SDS. Membranes were equilibrated in buffer A (100 mM Tris HCl, 150 mM NaCl; pH 7.5) for

Table 1. Finger millet lines from which DNA was extracted, the countries of origin and geographic regions

Number	Pedigree	Origin	Geographic Region
1.	SDFM 63	Zimbabwe	Southern Africa
2.	SDFM 77	Zimbabwe	Southern Africa
3.	25 C	Zimbabwe	Southern Africa
4.	SDFM 1708	Malawi	Southern Africa
5.	SDFM 1143	Malawi	Southern Africa
6.	SDFM 1706	Zambia	Southern Africa
7.	SDFM 1714	Zambia	Southern Africa
8.	SDFM 1545	Tanzania	East Africa
9.	SDFM 1547	Tanzania	East Africa
10.	SDFM 1072	Uganda	Lake Victoria
11.	SDFM 2545	Uganda	Lake Victoria
12.	SDFM 405	Zaire	Lake Victoria
13.	SDFM 406	Zaire	Lake Victoria
14.	SDFM 2543	Kenya	Lake Victoria
15.	SDFM 2544	Kenya	Lake Victoria
16.	SDFM 957	India	South India (Asia)
17.	SDFM 723	India	South India (Asia)
18.	ZFM-2316	Zambia	Southern Africa
19.	PR 404	India	North India (Asia)
20.	Thenga Murty	India	North India (Asia)
21.	IE-2929	Malawi	Southern Africa
22.	IE-2924	Malawi	Southern Africa
23.	ZFM-123	Zambia	Southern Africa
24.	ZFM-2103	Zambia	Southern Africa
25.	Mutubila	Zambia	Southern Africa
26.	L-197	Zambia	Southern Africa

1 minute, and then blocked by incubating in buffer B (2% w/v Boehringer-Mannheim blocking reagent dissolved in buffer A) for two hours at room temperature. Near the end of the blocking step, anti-digoxigenin alkaline phosphatase was diluted 1 : 5000 in buffer B for a working concentration of 150 mU/ml. The membranes were then transferred to the antibody conjugate solution and incubated for 30 minutes at room temperature with shaking, then the solution was discarded. The membranes were washed twice for 15 minutes in buffer A then sprayed on their DNA side with room temperature Lumi-Phos 530 before placement in transparent plastic bags. Membranes were incubated at 37° C for 30 minutes to allow light emission to reach a steady state, and then exposed to X-ray film for 15–45 minutes.

Membrane stripping

Membranes were reused for hybridization with a different probe by first rinsing in water, followed by incubation in 0.2 N NaOH, 0.1% SDS, at 37° C for 30 minutes with constant shaking. This procedure removed the digoxigenin labelled probe, while leaving the target nucleic acid bound to the membrane.

Results

Hybridization of finger millet total DNA Southern blots with three maize and one sorghum mitochondrial gene probes gave identical RFLP banding patterns in 23 of the 26 ecotypes from Asia and Africa. Three ecotypes, SDFM 1143, SDFM 77 and SDFM 63 showed polymorphisms.

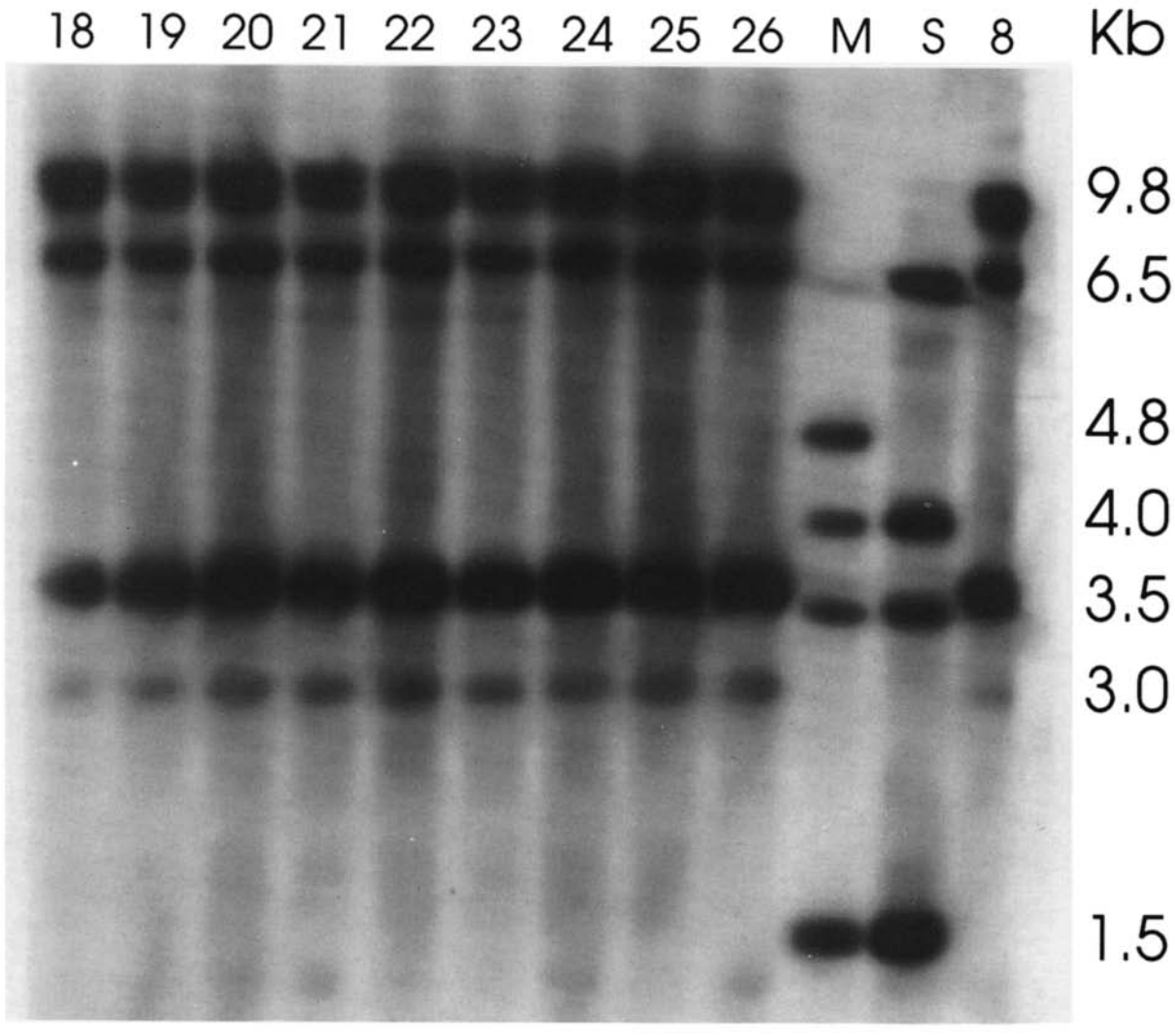

Fig. 1. Typical monomorphic patterns of a Southern blot containing *Hind*III digested DNA of finger millet ecotypes and maize and sorghum lines, hybridized to the *cox1* probe. Sizes of the fragments with homology to that probe are indicated. Lane numbers correspond to numbers in Table 1, except that lane S is sorghum and lane M is maize, respectively.

Monomorphic and polymorphic restriction fragment patterns

A typical restriction fragment hybridization banding pattern displayed by the 26 finger millet ecotypes for most of the enzyme/probe combinations is shown in Fig. 1 for the *Bam*H1/*cox1* illuminograph. The *cox1* probe hybridized to four distinct fragments ranging in molecular weight from 9.8 Kb to 3.0 Kb in all the finger millet ecotypes. Four hybridization bands were also observed for sorghum (lane S) and five in maize (lane M). The molecular weights of hybridized fragments ranged from 6.5 Kb to 1.5 Kb for both species. Each of the three species represented displayed unique banding patterns, indicating interspecific polymorphism. The *cox1* probe hybridized to 9.8 and 3.0 Kb fragments found only in finger millet; to a 4.8 Kb fragment found only in maize; and to 4.0 Kb and 1.5 Kb fragments in both sorghum and maize.

Table 2 summarizes the number and sizes of bands generated by each enzyme/probe combination for the finger millet lines. The number of hybridized fragments ranged from eight with the *Xba*1/*atp9* combination to two generated by five different probe/enzyme combinations. Only two enzyme/probe combinations revealed RFLPs among the finger millet lines (Table

Table 2. Finger millet Southern blot hybridization banding patterns (fragment sizes in Kb and numbers) generated by 5 enzymes × 4 probes (= 20) combinations

Band #	Enzyme																			
	Eco RI				Eco RV				Xba 1				Bam H1				Hind III			
	a[a]	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Kb																				
1.	6.3	2.5	9.0	9.5	9.9	4.0	20	9.9	15	9.5	19	20	20	9.5	10	6.5	3.8	9.8	9.9	8.5
2.	5.2	2.0	6.4	6.4	4.0	3.5	9.5	3.0	11	9.0	9.0	18	15	6.0	9.9	3.5	3.0	6.5	4.5	3.5
3a.										6.5[b]										
3.	3.5	1.5				2.5	4.0	1.0	9.9	6.4	6.5	8.0		5.0	6.4		1.0	3.5	3.0	3.0
4.						1.0	3.5		9.8	6.0	5.0	6.5		3.5	4.5			3.0	2.4	
5.						1.5	2.5		9.7	3.5	3.0	3.2			3.5				2.3	
6.									4.0	2.2										
6a.									3.6[c]						3.0					
7.									3.0	1.0										
8.									2.5											
Total #	3	3	2	2	2	5	5	3	8	7	5	5	2	3	6	2	3	4	5	3

[a] Probes: a = *atp9*, b = *cox1*, c = T *urf2B*, d = *18S-5S*.
[b] This 6.5 Kb fragment is found in two lines, SDFM 63 and 77 from Zimbabwe (see Fig. 3.).
[c] The 3.6 Kb fragment is found only in ecotype SDFM 1143 from Malawi, in conjunction with a missing 3.0 Kb fragment missing (see Fig. 2).

2). *Atp9* hybridized to an extra 3.6 Kb *Xba*1 fragment in ecotype SDFM 1143 from Malawi, but did not hybridize to a 3.0 Kb fragment present in all other ecotypes (Fig. 2, lane 5). The second polymorphism was observed in two Zimbabwean lines, SDFM 63 which was the only white seeded line, and SDFM 77. An extra, low intensity 6.5 Kb *Xba*1 band was detected by the *cox1* probe in both these lines (Fig. 3, lanes 1 and 2). These unique lines were not polymorphic in any other enzyme/probe combination.

Possible cytoptype groups

Based on the banding patterns generated by the 20 enzyme/probe combinations, the 26 lines can be classified into three cytotype groups. Group 1 consists of the 23 lines that were monomorphic across all enzyme/probe combinations. This could be considered the standard cytotype group. Group 2, represented by SDFM 1143, and group 3 cytotypes, represented by SDFM 63 and SDFM 77, respectively, appear to be subtypes of one basic cytotype group.

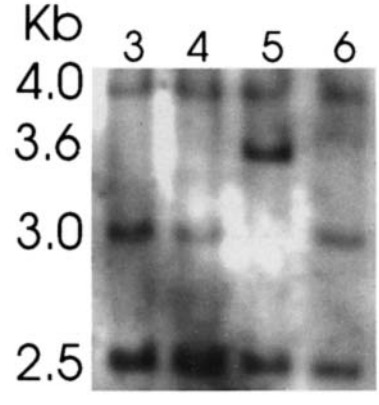

Fig. 2. Banding patterns of a Southern blot containing *Xba*1 digested DNA of finger millet ecotypes, hybridized to the *atp9* probe. Sizes of some of the fragments with homology to that probe are indicated. Lane numbers correspond to line numbers in Table 1. In lane 5 (SDFM 1143) the 3.0 Kb band is replaced by a 3.6 Kb band.

Discussion

The most prevalent result of this study was the high level of monomorphism apparent among the 26 finger millet ecotypes examined. Ninety percent (18 of 20) of the enzyme/probe combinations gave only one restriction

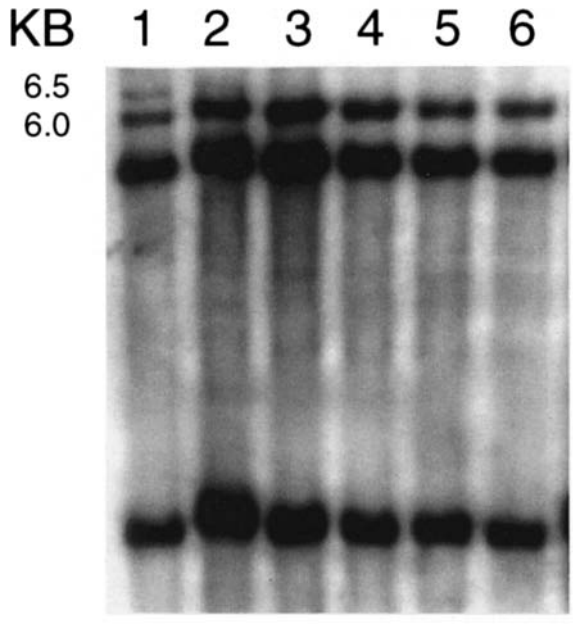

Fig. 3. Banding patterns of a Southern blot containing *Xba*1 digested DNA of finger millet ecotypes, hybridized to the *cox1* probe. Sizes of some fragments with homology to that probe are indicated. Lane numbers correspond to line numbers in Table 1. Lanes 1 (SDFM 63) and 2 (SDFM 77) show an extra 6.5 Kb band.

fragment pattern across the different entries. In addition, the polymorphisms detected were single variant fragments detected with enzyme/probe combinations which hybridized to seven nonvarient fragments. Consequently, the mitochondrial genome of these diverse finger millet ecotypes is highly conserved.

The nature of the monomorphic and polymorphic restriction fragment pattern profiles generated in this study was similar to those observed in studies on different crop species. The RFLPs observed here between finger millet, sorghum, and maize with all probe enzyme combinations reflects the high level of interspecific variation that exists in mtDNA. Intraspecific mtDNA variation, however, has been reported to occur on a much smaller scale (Gepts & Clegg, 1989). In pearl millet, mtDNA classification of three distinct cytoplasmic male sterile (CMS) groups was possible based on single fragment differences, detected by *Bam*HI/*atp9*, *Bam*HI/*cox1* and *Bam*HI/*18s-5s* (Smith & Showdhury, 1989). With each probe, only one polymorphic mtDNA fragment was identified among those hybridized. In the study reported here, *Xba*1/*atp9* and *Xba*1/*cox1* each identified a finger millet line with one unique mtDNA fragment. Monomorphic and polymorphic fragments with varying intensities were also observed in our study. Weihe et al. (1991) detected a minor hybridization signal to a mtDNA fragment with lower stoichiometry in sugar beet (*Beta vulgaris*) which was used to classify a subtype of the 'Owen' cytoplasm in this species. This result is similar to the lower intensity *Xba*1 fragment hybridized by *cox1* which identifies SDFM 63 and SDFM 77 as representing a unique cytoplasmic type. While the use of additional enzyme/probe combinations would likely reveal more mtDNA polymorphism in finger millet, this study demonstrates that the strategy used here was able to identify distinct cytoplasmic groups in a manner similar to that observed with other crop species.

The mtDNA variation detected in this study is the first report of molecular and cytoplasmic diversity within this crop species. Hilu (1988, 1992) found cpDNA and rDNA monomorphism among cultivated finger millet and the progenitor *E. coracana* spp. *africana*. The detection of cytoplasmic variation with mtDNA probes was consistent with the hypothesis that the mitochondrial genome is generally less conserved than cpDNA. The sampling of morphologically diverse lines representing the ecogeographic distribution of the crop in this study also aided in the detection of variation. Within the 26 lines selected, the most likely source of polymorphisms, however, would have been between the Indian and the African ecotypes because of the early isolation of the respective races, as early as second millenium B.C. (Hilu et al., 1979). Hussaini et al. (1977) measured morphological variation in vegetative and reproductive characters to identify twelve broad groups among 640 finger millet genetic stocks. Increasing the sampling to include more Asian lines or ecotypes representing every known race in finger millet would increase the chance of detecting mtDNA variation in future studies. A more extensive assessment of cytoplasmic variation would better describe the relationships among finger millet ecotypes. The evidence reported here, however, provides a means for the classification of finger millet lines into three cytoplasmic groups.

The ability to classify finger millet into cytoplasmic groups by mtDNA RFLPs could be a useful tool for breeders. Female parent selection in crossing programs could be based on this system to reduce reliance on a single cytoplasm. The recent identification of stable genetic male sterility in finger millet (Muza, 1993) makes this information more valuable as it now should be possible to readily generate hybrids with specific cytoplasms or transfer nuclear genetic variation into

unique cytoplasms and thus increase the cytoplasmic diversity among germplasm sources for this important crop species.

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References

Birnboim, H.C. & J. Doly, 1979. A rapid alkaline procedure for screening recombinant DNA. Nucleic Acids Res. 7: 1513–1515.

Borck, K.S. & V. Walbot, 1982. Comparison of the restriction endonuclease digestion patterns of mitochondrial DNA from normal and male sterile cytoplasms of *Zea mays* L. Genetics 102: 109–128.

Chowdhury, M.K.U. & R.L. Smith, 1988. Mitochondrial DNA variation in pearl millet and related species. Theor. Appl. Genet. 76: 25–32.

Feinberg, A.P. & B. Vogelstein, 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6–13.

Gengenbach, B.G. & J.A. Connelly, 1981. Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. Theor. Appl. Genet. 59: 161–167.

Gepts, P. & M.T. Clegg, 1989. Genetic diversity in pearl millet (*Pennisetum glaucum* L. R. Br.) at the DNA sequence level. Heredity 80: 203–208.

Hilu, K.W., 1988. Identification of the 'A' genome of finger millet using chloroplast DNA. Genetics 118: 163–167.

Hilu, K.W. & J.M.J. de Wet, 1976. Domestication of *Eleusine coracana*. Econ. Bot. 30: 199–208.

Hilu, K.W., J.M.J. de Wet & J.R. Harlan, 1979. Archeobotany and the origin of finger millet. Am. J. Bot. 66: 330–333.

Hilu, K.W. & J.L. Johnson, 1992. Ribosomal DNA variation in finger millet and wild species of *Eleusine* (Poacea). Theor. Appl. Genet. 83: 895–902.

Hussaini, S.H., M.M. Goodman & D.H. Timothy, 1977. Analysis and the geographical distribution of the world collection of finger millet. Crop. Sci. 17: 257–263.

Kemble, R.J., R.E, Gunn & R.B. Flavell, 1980. Classification of normal and male-sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. Genetics 95: 451–458.

Kemble, R.J., R.E. Gunn & R.B. Flavell, 1983. Mitochondrial DNA variation in races of maize indigenous to Mexico. Theor. Appl. Genet. 65: 129–144.

Khairallah, M.M., M.W. Adams & B.B. Sears, 1991. Mitochondrial genome size variation and restriction fragment length polymorphisms in three *Phaseolus* species. Theor. Appl. Genet. 82: 321–328.

Levings, III, C.S. & D.R. Pring, 1977. Diversity of mitochondrial genomes among normal cytoplasms of maize. J. Hered. 68: 350–354.

McNay, J.W., D.R. Pring & D.M. Lonsdale, 1983. Polymorphism of mitochondrial DNA 'S' regions among normal cytoplasms of maize. Plant Mol. Biol. 2: 177–187.

Muza, F.R., 1993. Mutagenesis and RFLP applications in finger millet (*Eleusine coracana* L. Gaertn.) breeding. Ph.D. Thesis. University of Nebraska, Lincoln.

Palmer, J.D., 1985. Evolution of chloroplast and mitochondrial DNA in plants and algae. p. 131–240. In: R.J. McIntyre (Ed). Monograph in Evolutionary Biology: Molecular evolutionary Genetics. Plenum Press, New York.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen & R.W. Allard, 1984. Ribosomal DNA spacer polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014–8018.

Smith, R.L. & M.K.U. Chowdhury, 1989. Mitochondrial DNA polymorphism in male-sterile and fertile cytoplasms of pearl millet. Crop Sci. 29: 809–814.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503–517.

Weihe, A., N.A. Dudareva, S.G. Veprev, S.I. Maletsky, R. Melzer, R.I. Salganik & Th. Borner, 1991. Molecular characterization of mitochondrial DNA of different subtypes of male-sterile cytoplasms of the sugar beet *Beta vulgaris* L. Theor. Appl. Genet. 82: 11–16.

Weissinger, A.K. & M.C. Albertson, 1984. Measurement of cytoplasmic variation and its applications in corn breeding. p. 111–122. In: R.Brown & D. Wilkinson (Ed). Proc. Annu. Corn. Sorghum Res. Con., 39th, Chicago II. 5–6 Dec. 1984. Am. Seed Trade Assoc., Washington, D.C.