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RFLP analysis of cytoplasmic male-sterile lines of pigeonpea [*Cajanus cajan* (L.) Millsp.] developed by interspecific crosses

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Summary

Total DNA from three putative cytoplasmic male sterile (CMS) progenies derived from crosses between the wild species *Cajanus sericeus* and the cultivated species *Cajanus cajan*, five *C. cajan*, one accession of *C. sericeus* and two genetic male sterile lines of pigeonpea were compared for their RFLP patterns using maize mitochondrial DNA (mtDNA) specific probes. Three putative cytoplasmic male sterile (CMS) progenies from the multiple cross genome transfer of pigeonpea lines (CMS 7-1, CMS 12-3, and CMS 33-1) showed hybridization patterns identical to that of *C. sericeus* when DNA was digested with *Eco*RI and *Hind*III and probed with maize mtDNA clones. The results suggested that these putative CMS progenies have the mitochondria of the female wild species parent. The hybridization patterns of the three male parental lines used in the development of the CMS progenies were similar in all the restriction enzyme-probe combinations except *Hind*III-*atp*6. The genetic male sterile lines, MS Prabhat and QMS 1 differed from each other in their hybridization pattern. The genomic DNA hybridization pattern of *Hind*III digested DNA from ICPL 87 differed from the other pigeonpea lines when probed with the maize mtDNA clones. The cluster analysis of the hybridization data suggested the occurrence of variation in the mitochondrial genome even among the cultivated species.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is one of the important legume crops in the tropics and subtropics (Nene and Sheila, 1990). The crop is grown in different cropping systems and for multiple uses (food, fodder and fuel) in many parts of the semi-arid tropics of India and Africa (Laxman Singh et al., 1990). Pigeonpea is a partially cross-pollinated crop with over 20% cross pollination. The outcrossing nature of pigeonpea is being exploited for hybrid breeding using stable genetic male sterility discovered by Reddy et al. (1978). Commercial production of pigeonpea hybrid seeds using the genetic male sterile lines poses the problem of roguing plants from the plots. The alternative has been to look for cytoplasmic male sterile (CMS) lines which have been successfully exploited in several crops. In crop plants, it is of great economic importance to have CMS for commercial hybrid production. Attempts to obtain cytoplasmic male sterility in spontaneous mutants in the world pigeonpea collection have been unsuccessful (Reddy et al., 1978). Indications of the presence of a CMS system has been reported by Ariyanayagam et al. (1995) in the progenies of the crosses between wild species *C. sericeus* and cultivated *C. cajan*.

Cytoplasmic male-sterility (CMS) is a maternally inherited trait with the plant remaining female fertile but with no viable pollen formation. This results from incompatible nuclear-mitochondrial interaction due to rearrangements in the mitochondrial genome (Lonsdale, 1987). Genetic and molecular methods have been used to study the differences in the organization and expression of the mitochondrial genes in the fertile/sterile phenotypes (Hanson and Conde, 1985). Restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) has been used as an efficient tool to look at rearrangements in the mtDNA of male sterile lines in many crop species (Boeshore et

al., 1983; Khairallah et al., 1991; Smith and Chowdhury, 1989; Bailey-Serres et al., 1986; Weihe et al., 1991). The technique has also been used to look at the differences between male sterile and their counterpart maintainer lines and for classification of the different cytoplasmic systems (Pring et al., 1982; Rajeshwari et al., 1994). The RFLP and RAPD (Random Amplified Polymorphic DNA) analyses have been used to arrive at the phylogenetic relationship among the cultivated and wild species of pigeonpea (Nadimpalli et al., 1993; Ratnaparkhe et al., 1995). In the present study we have carried out a RFLP analysis of total DNA from the different male parents of *Cajanus* species, the wild species *C. sericeus* used as the female parent and the putative CMS progenies.

Materials and methods

Plant material

The pigeonpea cultivars were grown in pots in a greenhouse at 25 °C as described by Ariyanayagam et al. (1995). The first cross for developing a cytoplasmic system of male-sterility was made between *C. sericeus* accession EC 121208, as female parent, and *C. cajan* line ICPX 880227, as the male parent. Transfer of the nuclear genome of pigeonpea into *C. sericeus* cytoplasm was affected through successive genomic transfer stages (GTS) with different male parents of pigeonpea instead of the conventional backcrossing as described by Ariyanayagam et al. (1995). Genome transfer stages require backcrossing with more than one pigeonpea genotype and this approach differs from the conventional backcrossing where substitution of the genome of a specific genotype is finally desired. This mode of transfer is often referred to as multiple cross genome transfer. The other male parents used in the mating program were ICPL 90035, ICPL 85030 and ICPL 85010. Three selected male-sterile lines derived from the sixth genetic transfer stage (GTS 6) were: 7-1 (*C. sericeus* × ICPX 880227 × 90035 × 85030 × 85030 × 85030) × 85030, 12-3 (*C. sericeus* × ICPX 880227 × 90035 × 85030 × 85030) × 85030, and 33-1 (*C. sericeus* × ICPX 880227 × 90035 × 85030 × 90035) × 85010. These lines designated as CMS 7-1, CMS 12-3, and CMS 33-1 had an average pollen sterility of 85%, 70% and 75% respectively. The cultivar ICPL 87 was used as the standard check for the male parents and the two genetic male sterile lines MS Prabhat and QMS 1 having different male sterility genes (Wallis et al., 1980) were included for comparison.

DNA extraction

DNA was extracted from pigeonpea leaves as described by Murray and Thompson (1980) with some modifications. Fresh leaf (3–4 g) was ground in liquid Nitrogen and the powder was transferred to a 50 mL polypropylene tube containing 20 mL of extraction buffer (2% CTAB, 0.2 M Tris-HCl, pH 8.0, 1.4 m NaCl, 0.02 M EDTA and 2% mercaptoethanol). The contents of the tube were gently mixed by inversion and incubated in a waterbath at 65 °C for 1 h. After cooling to room temperature an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the contents were mixed gently for 5 min by inversion and the layers were separated by centrifugation. The aqueous layer was removed and the above step was repeated one more time. DNA was precipitated by the addition of an equal volume of isopropanol and was hooked out with a bent Pasteur pipet. DNA was washed twice with 70% ethanol and dissolved in TE (0.05 M Tris-HCl pH 8.0, 0.010 M EDTA). Ribonuclease was added to a final concentration of 50 μg/ml and incubated at 37 °C for 1 h. An equal volume of phenol:chloroform (1:1 v/v) was added and mixed for 5 min by inversion. The aqueous layer was removed after centrifugation and an equal volume of chloroform:isoamyl alcohol (24:1) was added and extracted as before. DNA was precipitated by the addition of 0.3 M sodium acetate and an equal volume of cold isopropanol. The DNA pellet was washed twice with 70% ethanol and redissolved in TE (0.01 M Tris-HCl pH 8.0, 0.001 M EDTA).

Clones used

The maize *atp6* clone (ATPase subunit 6, Dewey et al., 1985a), and *atp9* (Dewey et al., 1985b) was supplied by C.S. Levings III, Genetics Department, North Carolina State University, Raleigh, NC, USA. Maize clones, *cox*I (Cytochrome oxidase subunit I, Isaac et al., 1985), *cox*II (Cytochrome oxidase subunit II, Fox & Leaver, 1981) and *atp*α (Isaac et al., 1985) were provided by C.J. Leaver, Department of Plant Science, University of Oxford, Oxford, U.K.

DNA analysis

Total DNA (about 10 μg) was digested with 30 units of the restriction enzymes *Eco*RI; *Hind*III, and *Eco*RV

as per the manufacturer's protocols and the fragments were separated on 0.8% agarose gels by electrophoresis in TBE buffer (0.089 M Tris-Borate 0.002 M EDTA, pH 8.0). The fragments were viewed under UV light after staining with ethidium bromide (0.5 μg/ml). After washing the gels in water the DNA fragments were transferred onto Nylon membrane (Amersham, UK) by vacuum transfer. DNA was bonded to the membrane by exposure to UV light as per the manufacturer's protocol (Stratagene).

The random primed labeling method of Feinberg and Vogelstein (1983) was used for the preparation of ^{32}P-labeled probes. Southern blots were prehybridized with 30 mL of pre-hybridization solution containing 7% SDS, 1% BSA, 0.5 M Na_2HPO_4 and 20 μg/mL sheared and denatured salmon sperm DNA per two blots (20 $\times$ 15 cm) size. Hybridization was carried out by adding the labeled probe to the prehybridization solution and incubating for 16 h at 65 °C in standard bottles (30 $\times$ 3.5 cm) in a hybridization oven (Hybaid, U.K.). The blots were washed three times in 3$\times$SSC containing 0.1% SDS at 65 °C for 30 min each. Autoradiography of the blots was conducted at -70 °C for varying periods using X-AR (Kodak) films. The fragment sizes were determined using lambda DNA cut with *Hind*III standard markers.

Similarity index matrices were generated based on the proportion of common restriction digestion fragments between two accessions (Nei, 1987) using

$$F = \frac{2M_{xy}}{M_x + M_y}$$

where 'F' is de similarity index, M_x is the number of bands in accession x, M_y is the number of bands in accession y, and M_{xy} is the number of bands common to both x and y. Cluster analysis of data was carried out using the statistical software package GENSTAT and a dendrogram was constructed.

Results and discussion

Southern blot hybridization of *Eco*RI digested total DNA when probed with the maize mtDNA specific *atp*6 clone revealed identical patterns for *C. sericeus* and the three putative CMS progenies obtained after the multiple genome transfer of pigeonpea lines, CMS 7-1, CMS 12-3, and CMS 33-1 (Figure 1, lanes 4–7), and these were distinct from all the other male parents used in the crosses and the genetic male-sterile lines studied. Three of the male parents used in the crosses

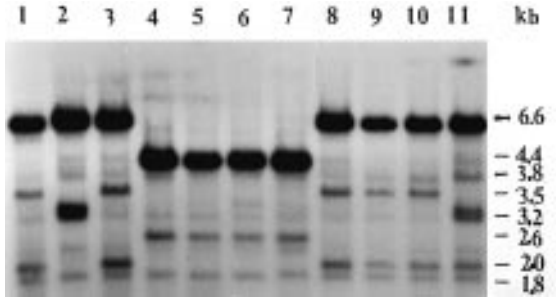

Figure 1. Southern blot hybridization of genomic DNA from putative male-sterile and the parental lines of pigeonpea digested with *Eco*RI and probed with maize *atp*6 clone. Lanes 1: ICPL 87, 2: MS Prabhat, 3: QMS 1, 4: CMS 7-1, 5: CMS 12-3, 6: CMS 33-1, 7: *C. sericeus*, 8: ICPX 880227, 9: ICPL 85030, 10: ICPL 90035 and 11: ICPL 85010. Fragment sizes are indicated in kilobases.

and ICPL 87 showed identical hybridization patterns (Figure 1, lanes 8–10) whereas ICPL 85010 (Figure 1, lane 11) showed a different pattern. The genetic male-sterile lines, MS Prabhat (Figure 1, lane 2) differed from the genetic male sterile line QMS 1 (Figure 1, lane 3) and other male parental lines in the hybridization patterns. The two genetic male sterile lines MS Prabhat and QMS thus do differ in the mitochondrial genome, besides their differences that contribute to sterility (Saxena et al., 1986). ICPL 85010 showed a pattern (Figure 1, lane 11) similar to that of MS Prabhat (Figure 1, lane 2) though there were some differences in the weakly hybridizing bands. DNA when digested with *Hind*III and probed with *atp*6 gave different hybridization patterns for each of the male parental lines which was mainly based on the weakly hybridizing bands (data not shown).

Other maize mtDNA clones (*atp*α, *atp*9) gave identical results with pigeonpea genomic DNA from the above lines though the patterns of hybridization were different from that of *atp*6 (Table 1). On the other hand, the maize mtDNA clone *cox*I could only distinguish the wild species and the putative CMS lines from the other pigeonpea cultivars and ICPL 87 by forming three groups (Table 1). Southern blot hybridization of pigeonpea DNA with the maize *cox*I probe showed only three groups which is in contrast to the results with the other maize probes and that obtained with *Eco*RI (Table 1).

The three putative male-sterile lines CMS 7-1, CMS 12-3, and CMS 33-1 showed hybridization patterns identical to that of *C. sericeus* when *Hind*III digested total DNA was probed with the maize *atp*9 clone (Figure 2, lanes 4–7). The three male parental

Table 1. Size differences of pigeonpea DNA fragments (kb) hybridized to maize mtDNA clones

Restriction enzyme-probe combination	Group I	Group II	Group III	Group IV
Enzyme: *Eco*RI				
*atp*9	8.2, 4.2, 2.6	9.2, 6.5, 4.2	9.2, 6.5, 4.2	
	1.0, 0.5	3.2, 1.0	2.9, 1.0	
atpα	4.8, 2.1, 0.5	3.1, 2.1	2.5, 2.1	
*cox*I	8.0, 7.4, 6.0	11.9, 8.0, 7.4		
	4.9, 3.9, 3.2	4.9, 3.9, 3.2		
Enzyme: *Hind*III				
*atp*6	8.9, 4.5	16.1, 4.5, 4.2	16.1, 4.2,	16.1, 5.5, 4.2
		4.0, 2.4	2.9, 2.4	4.0, 2.4
atpα	12.2, 11.5	4.2, 3.5	2.9	5.9, 4.2, 3.5
	10.9, 4, 4.5			
*cox*I	5.6, 4.6	6.6, 6.2, 4.4		6.6, 6.2
	4.4, 3.7			4.4, 2.8

Group I: *C. sericeus*, 3 putative CMS lines.
Group II: 3 male parental lines and QMS 1.
Group III: MS Prabhat and ICPL 85010.
Group IV: ICPL 87.
In *Eco*RI-*cox*I, group II includes group III and group IV cultivars.
In *Hind*III-*cox*I, group II includes all group III cultivars.

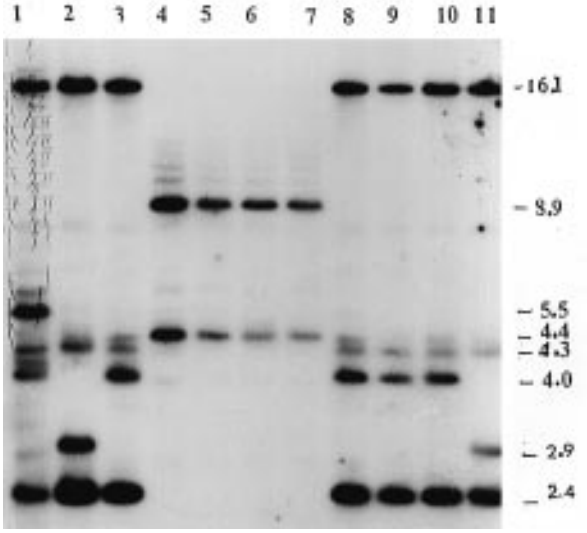

Figure 2. Southern blot hybridization of genomic DNA from putative male-sterile and the parental lines of pigeonpea digested with *Hind*III and probed with maize *atp*9 clone. Lanes 1: ICPL 87, 2: MS Prabhat, 3: QMS 1, 4: CMS 7-1, 5: CMS 12-3, 6: CMS 33-1, 7: *C. sericeus*, 8: ICPX 880227, 9: ICPL 85030, 10: ICPL 90035 and 11: ICPL 85010. Fragment sizes are indicated in kilobases.

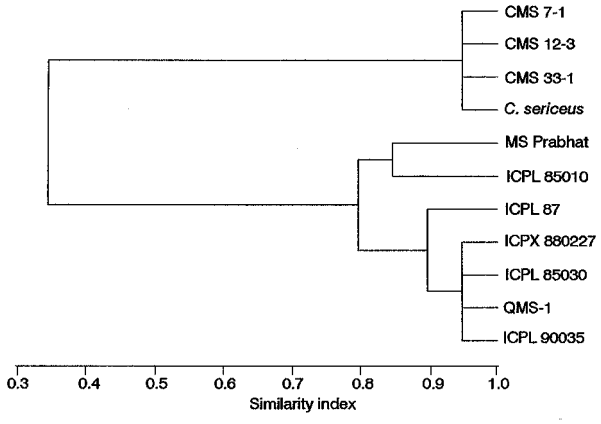

Figure 3. Dendrogram of putative male-sterile and the parental lines of pigeonpea based on mtDNA probes hybridized to genomic DNA. Cluster analysis of the data obtained from the hybridization of *Eco*RI and *Hind*III-digested pigeonpea genomic DNA with the maize probes *atp*6, *atp*9, *atpα*, and *cox*I was done as described in the Materials and methods.

lines ICPX 880227, ICPL 95030 and ICPL 90035 and the genetic male sterile line QMS 1 (Figure 2, lanes 8–10 and 3 respectively) showed identical hybridization patterns. The two genetic male sterile lines, MS Prabhat and QMS 1 (Figure 2, lanes 2 and 3) differed from each other in their hybridization patterns. The results obtained with this enzyme-probe combination

were similar to those obtained with *EcoR*I digested DNA except that ICPL 87 could be distinguished from all other male parental lines by the presence of the additional 5.5 kb fragment which is absent in the other lines (Figure 2, lane 1). Similar results were obtained with other maize mtDNA probes (Table 1). The differrences in DNA hybridization patterns between ICPL 87 and other *C. cajanus* line further suggests that even within the cultivated *Cajanus* species there is variation in the mitochondrial genome, though the reasons for the differences are not clear. The dendrogram based on the cluster analysis of the hybridization data put the putative male-sterile lines and the wild species *C. sericeus* into one major group and the rest of the parental lines and genetic male sterile lines into another group (Figure 3). In the other major group three subgroups could be identified: 1) MS Prabhat and ICPL 85010, 2) ICPL 87 and 3) the rest of the male parental lines (Figure 3).

The results presented here thus suggest that the crosses between the wild species *C. sericeus* and the cultivated species *C. cajan* produced male-sterile lines having the mitochondria of the former. The differences in the restriction fragment patterns among the various pigeonpea cultivars could be the result of inter- and intra-molecular rearrangements within the mitochondrial genome, which is a common phenomenon in higher plant mitochondria (Lonsdale et al., 1988; Levings and Brown, 1989).

The results from the cluster analysis suggest that even among the cultivated species there can be variation in the mitochondrial genome as evident in the differential hybridization patterns of ICPL 87 and other male parental lines. It is assumed that mitochondria and chloroplast genomes are mostly conserved among species though mitochondrial genome is known to show large variation due to recombination events among the number of repeat sequences present. It would be interesting to see the differences in the mitochondrial genome among the cultivars of cultivated species.

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References

Ariyanayagam, R.P., A. Nageshwar Rao & P.P. Saveri, 1995. Cytoplasmic-genic male-sterility in interspecific matings of *Cajanus*. Crop Sci 35: 981–985.

Bailey-Serres, J., L.K. Dixon, A.D. Liddell & C.J. Leaver, 1986. Nuclear mitochondrial interactions in cytoplasmic male-sterile sorghum. Theor Appl Genet 73: 252–260.

Boeshore, M.L., I. Lifshitz, M.R. Hanson & S. Izhar, 1983. Novel composition of mitochondrial genomes in Petunia somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190: 459–467.

Dewey, R.E., C.S. Levings III & D.H. Timothy, 1985a. Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. Plant Physiol 79: 914–919.

Dewey, R.E., A.M. Shuster, C.S. Levings III & D.H. Timothy, 1985b. Nucleotide sequence of Fo-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc Natl Acad Sci 82: 1015–1019.

Feinberg, A.P. & B. Vogelstein, 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137: 266–267.

Fox, T.D. & C.J. Leaver, 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26: 315–323.

Hanson, M.R. & M.F. Conde, 1985. Functioning and variation of cytoplasmic genomes: lessons from cytoplasmic-nuclear interactions affecting male fertility in plants. Int Rev Cytol 94: 213–267.

Isaac, P.G., V.P. Jones & C.J. Leaver, 1985. The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male-sterile plants. EMBO J 4: 1617–1623.

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.

Laxman Singh, S.C. Gupta & D.G. Faris, 1990. Pigeonpea: Breeding. In: Y.L. Nene, S.D. Hall & V.K. Sheila (Eds) Pigeonpea. C.A.B. International. pp 375–400.

Levings, C.S. III & G.G. Brown, 1989. Molecular biology of plant mitochondria. Cell 56: 171–179.

Lonsdale, D., 1987. Cytoplasmic male sterility: A molecular perspective. Plant Physiol Biochem 25: 256–271.

Londsdale, D.M., T. Brears, T.P. Hodge, S.E. Melville & W.H. Rottmann, 1988. The plant mitochondrial genome: homologous recombination as a mechanism for generating heterogeneity. Philo Trans R Soc Lond Ser B 319: 149–163.

Murray, M.G. & W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8: 4321–4325.

Nadimpalli, R.G., J.L. Jarret, S.C. Phatak & G. Kochert, 1993. Phylogenetic relationships of the pigeonpea (*Cajanus cajan*) based on nuclear restriction fragment length polymorphisms. Genome 36: 216–223.

Nei, M., 1987. Molecular evolutionary genetics. Columbia University Press New York.

Nene, Y.L. & V.K. Sheila, 1990. Pigeonpea: Geography and importance. In: Y.L. Nene, S.D. Hall & V.K. Sheila (Eds) The Pigeonpea. C.A.B. International, U.K. pp 1–14.

Pring, D.R., M.F. Conde & K.F. Schertz, 1982. Organelle genome diversity in sorghum: male sterile cytoplasms. Crop Sci 22: 414–421.

Rajeshwari, R., S. Sivaramakrishnan, R.L. Smith & N.C. Subrahmanyam, 1994. RFLP analysis of mitochondrial DNA from cytoplasmic male-sterile lines of pearl millet. Theor Appl Genet 88: 441–448.

Ratnaparkhe, M.B., V.S. Gupta, M.R. Ven Murthy & P.K. Ranjekar, 1995. Genetic fingerprinting of pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives using RAPD markers. Theor Appl Genet 91: 893–898.

Reddy, B.V.S., J.M. Green & S.S. Bisen, 1978. Genetic male sterility in pigeonpea. Crop Sci 18: 362–364.

Saxena, K.B., D.G. Faris, L.J. Reddy, D. Sharma, B.V.S. Reddy, S.C. Gupta & J.M. Green, 1986. Prospects for hybrid pigeonpea. In: B. Napompeth & S. Subhadrabandhu (Eds) New frontiers in breeding researches. Proceedings of the Fifth International Congress organized by the Society for the Advancement of Breeding Researches in Asia and Oceania (SABRAO). Kasetsart University, Bangkok, Thailand, pp 379–385.

Smith, R.L., M.K.U. Chowdhury & D.R. Pring, 1987. Mitochondrial DNA rearrangements in *Pennisetum* associated with reversion from cytoplasmic male-sterility to fertility. Plant Mol Biol 9: 277–286.

Smith, R.L. & M.K.U. Chowdhury, 1989. Mitochondrial DNA polymorphisms in male-sterile and fertile cytoplasms of pearl millet. Crop Sci 229: 809–814.

Wallis, E.S., K.B. Saxena & D.E. Byth, 1980. A new source of genetic male sterility in pigeonpea. In: Y.L. Nene & V. Kumble (Eds) Intl crops research institute for the semi-arid tropics, vol. 2. Proc. Intl. workshop on pigeonpea. ICRISAT Center, Patancheru, India. 1980. pp 105–108.

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