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Mitochondrial and total DNA RAPD patterns can distinguish restorers of CMS lines in sorghum

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Abstract Seven sorghum restorer lines that differentially restore (or maintain) the A1 and A2 cytoplasmic male-sterile (CMS) cytoplasms were studied by RFLP analyses of their mtDNAs and RAPD analyses of their mitochondrial DNA (mtDNA) and total DNA to understand nuclear mitochondrial combinations that are present in these lines. RFLP data from 11 mitochondrial gene probes were inadequate to classify these seven lines. However, the analysis of RAPD profiles of total DNA could distinguish these lines on the basis of their ability to restore completely or partially the fertility in the A1/A2 CMS cytoplasms. Interestingly, RAPD profiles of mtDNAs of these lines also followed the same pattern as that of the total DNA. These results indicate that the different restorer lines possess specific nuclear-cytoplasm combinations. Further, the results also show that the RAPD technique can be used to identify markers for different cytoplasms used in CMS.

Key words Sorghum · RAPD · CMS · Fertility restoration · MtDNA

Introduction

Hybrid seed production in crop plants requires a cytoplasmic male-sterile (CMS) line (A) which is used as a female parent, a maintainer line (B) which is isonuclear with respect to the male-sterile line and a restorer line which not only restores fertility in the male-sterile (A) line but also produces seeds with hybrid vigour. Such three-line systems have been successfully developed for several crop plants including maize, brassica, rice and sorghum (Kaul 1988). Ever-increasing information on these systems has, over the years, led to the conclusion that maintenance of cytoplasmic male sterility, as also its restoration, are due to mitochondrial and nuclear genome interactions (Hanson 1991).

An understanding of CMS at the molecular level is now possible with the newer tools of molecular biology. Plant breeders, while evolving appropriate restorers for hybrid seed production, have identified genotypes that yield restoration either fully or partially. Such genotypes along with the maintainers and male-sterile lines provide excellent material for probing the molecular basis of sterility or fertility.

The different CMS lines available in plants can be considered as mitochondrial mutations wherein the change is corrected/regulated by the nuclear fertility restorer genes. The extent to which the nucleus is able to regulate the expression or correct the change of the mitochondrial CMS-related genes determines whether or not there will be complete, partial or a lack of fertility restoration. Male sterility and fertility can thus be considered to occur as a result of close interactions (either incompatible or compatible) between the cytoplasm and the nucleus not only in the CMS and fertility-restored plants but also in maintainers and restorers. While a lot of effort has gone into studying the genetics and molecular basis of cytoplasmic male sterility in different crops (Laughnan and Gabay-Laughnan 1983; Stephens and Holland 1954; Nagur and Menon 1974; Rao et al. 1984; Bharaj et al. 1991; Li and Yuan 1986, Dewey et al. 1986, 1987; Young and Hanson 1987, Moneger et al. 1994; Abad et al. 1995), very little information is available on the characteristics of maintainer and restorer cytoplasms at the molecular level. We have been interested in the molecular mechanisms

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of nuclear-cytoplasmic interactions, with particular reference to the sorghum and rice CMS systems. In the studies described herein we have used sorghum for which well-documented male-sterile, maintainer and restorer lines are available. Although several male-sterile cytoplasms are known, for example, A1, A2, A3, A4, A5, A6 etc. (Stephens and Holland 1954; Rao 1962; Hussaini and Rao 1964; Webster and Singh 1964; Nagur and Menon 1974; Schertz and Ritchey 1978; Pring et al. 1982; Lee et al. 1989; Xu et al. 1995), only the A1 (milo) cytoplasm has been used commercially for hybrid seed production. In our earlier studies (Sane et al. 1994, 1996), we reported the characterization of mitochondrial genomes of male-sterile lines, maintainer lines and restorer lines that fully restore sterility due to A1 cytoplasm, using restriction fragment length polymorphism (RFLP) and biochemical analysis. These studies indicated that RFLP can distinguish sterile, maintainer and restorer lines to some extent. In the study reported here, we used the random amplified polymorphic DNA (RAPD) technique to study if this method, which scans a much larger portion of the genome than the RFLP technique, can distinguish differences not only among male-sterile lines, maintainers and complete restorers but also the partial restorers of A1 and A2 cytoplasms. The mitochondrial genome analyses were complemented by nuclear genome analyses of these lines using the RAPD technique. Besides bringing out differences in mitochondrial genomes of restorers, these studies demonstrate that the sorghum restorer lines are characterized by specific nucleus-cytoplasm combinations.

Materials and methods

Plant material

The seeds of seven sorghum lines were generously provided by Dr. A. R. Dabholkar, AICSIP, JNKVV, Indore, India. Of the seven lines, six, CS3541, IS-23183, IS-1066, IS-40575, RS-29, ICSV-89058, are the complete or partial restorers for the A1 and A2 CMS cytoplasms while IS-3436 is a maintainer. The restoration efficiency of the six lines was investigated under field conditions at the College of Agriculture, Indore (under the All India Co-ordinated Sorghum Improvement Project), by Dr. A. R. Dabholkar in 1993 and is provided in Table 1. Mitochondrial gene probes for *atp*A and *cox*I were kindly provided by Prof. C. J. Leaver (UK) while those for *atp*6, *atp*9 and *cox*II were provided by Prof. C. S. Levings III (USA). Oligonucleotide probes for *nad*2, *nad*3, *rps*12, *rps*13, *cox*III and *orf*156 were kindly provided by Dr. J. M. Grienenberger (France).

Extraction of DNA and PCR conditions

Total genomic DNA was isolated from 7-day-old etiolated seedlings according to the method of Dellaporta et al. (1983), and mitochondrial (mt) DNA was isolated as described by Nath et al. (1993), except that the CsCl gradient step was excluded. In order to minimize plastid DNA contamination, we used etiolated seedlings for isolation of mtDNA. Moreover, the pellet, enriched for mitochondria after differential centrifugation, was treated with DNase to remove extra-mitochondrial (nuclear) DNA.

Amplification reactions were performed in 25-µl volumes according to Williams et al. (1990) and contained 25 ng of template DNA (mitochondrial/total genomic), 1 × *Taq* polymerase buffer, 10 pmol of primer, 0.2 m*M* each of the four dNTPs (Pharmacia) and 1 U *Taq* polymerase enzyme (Bangalore Genei, Bangalore, India). The amplification was carried out in a DNA Robocycler (Stratagene GmbH, Germany) programmed for 44 cycles of 60 s at 94°C, 90 s at 35°C and 120 s at 72°C, with a final cycle at 72°C for 5 min. Amplified products were analysed on 1.2% agarose gels in 1 × TAE buffer (Sambrook et al. 1989) and were detected by ethidium-bromide staining. The 60 primers used for RAPD analysis included 20 primers each of the kits OP-A, OP-B and OP-C from Operon Technologies (USA).

Table 1 Ability of the seven sorghum restorer lines to restore (or maintain) the A1 and A2 CMS cytoplasms. Experiments were carried out in the field at the College of Agriculture, Indore (A.R. Dabholkar, personal communication)

	Sorghum	Remarks
1	CS3541	Restores A1 cytoplasm completely
2	IS23183	Restores A1 cytoplasm completely
3	IS1066	Completely restores A1 and A2 cytoplasms
4	IS40575	Completely restores A1 and A2 cytoplasms
5	RS29	Restores A1 cytoplasm completely and A2 partially
6	ICSV89058	Restores A1 cytoplasm completely and A2 partially
7	IS3436	Maintains both A1 and A2 cytoplasms

Mitochondrial DNA RFLP

MtDNA (2 µg each) from the seven sorghum lines was digested with *Hin*dIII restriction enzyme (New England Biolabs, USA), and a Southern blot was raised on nylon membrane (Zetaprobe, BioRad Inc. USA). The blot was probed successively with five heterologous mitochondrial gene probes, *atp*A, *atp*6, *atp*9, *cox*I and *cox*II and six oligonucleotide probes for mitochondrial genes, *nad*2, *nad*3, *rps*12, *rps*13, *cox*III and *orf*156. Gene probes were radiolabelled with α-[^{32}P]dATP by random priming, while oligonucleotide probes were endlabelled with γ-[^{32}P]ATP as described by Sambrook et al. (1989). The hybridizations and autoradiography were carried out as per the instructions of the nylon membrane manufacturer.

Data analysis

The estimate of F values as an index for pairwise genetic similarity was calculated using the algorithm of Jaccard (1901) in the RAPDISTANCE package (ver 1.03, Armstrong et al. 1994). The pairwise similarity was based upon the cumulative matrix generated for all bands with all primers and probes used to obtain the RAPD and RFLP profiles respectively, considering a score of 1 when the DNA band was present and 0 when absent. The SAHN clustering of these data for the seven sorghum lines was done employing the UPGMA method of Sokal and Sneath (1963). Further, for the purpose of comparisons, the mean dissimilarity value was calculated as (1-F), where F is the similarity value calculated according to Jaccard (1901).

Results

Mitochondrial DNA RFLP analysis

Of the 11 probes used for generating RFLP profiles in the seven sorghum lines only 5, *atp*A, *atp*6, *atp*9, *nad*3 and *rps*12, revealed polymorphism. The 11 probes for the mitochondrial genome resulted in 22 DNA fragments, of which 45.5% were polymorphic (Table 2). The mean dissimilarity value (1-F) obtained from 28 pairwise comparisons for the seven genotypes was 0.104. Polymorphism in *atp*A, *atp*6, *atp*9, *nad*3 and *rps*12 could distinguish the A1 restorer CS3541 from all the other lines, while the complete A1-A2 restorer, IS40575, could be distinguished from all the other lines on the basis of its *atp*9 pattern (Fig. 1A). The rest of the lines did not show any polymorphism among themselves with any of the other mitochondrial probes. The findings were easily represented in the form of a dendrogram (Fig. 1B) obtained by UPGMA analysis based on the similarity index (F) values. The dendrogram indicates that the seven lines can only be clustered in three groups based on the RFLP analysis of their mitochondrial genome.

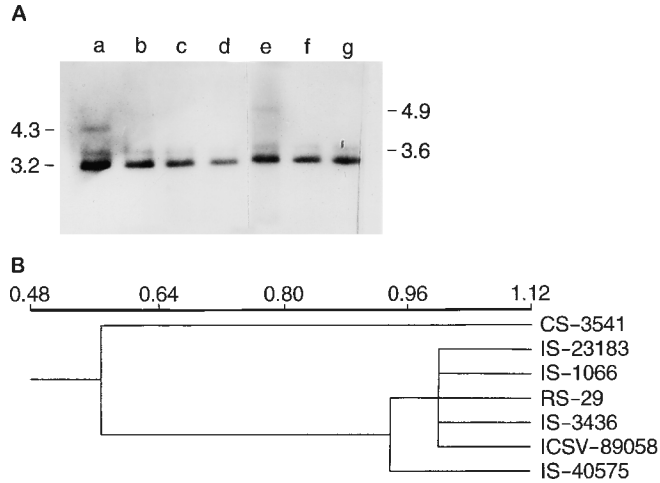

Fig. 1 **A** Southern blot analysis of the *Hin*dIII-digested mtDNAs of the seven restorer lines probed with *atp*9. *Numbers* on the *left* and right of the panels indicate sizes in kilobasepairs. *Lanes* marked *a–g* are CS3541, IS23183, IS1066, RS29, IS40575, ICSV89058 and IS3436, respectively. **B** UPGMA dendrogram generated from the RFLP data of the seven sorghum restorer mtDNAs

RAPD analysis of mtDNA

Sixty RAPD primers (arbitrary sequence decamers) were chosen to analyse the mitochondrial genomes of the seven sorghum lines. Of these, 14 primers did not result in profiles, while 16 primers were inconsistent or did not amplify fragments from all templates. Of the remaining 30 primers, RAPD profiles with 17 primers were selected for analysis and resulted in a total of 120 bands of which 71.66% were polymorphic. The mean dissimilarity value (1-F) from 28 pairwise comparisons was 0.282 (Table 3). Only OPA-18 generated a single monomorphic band while the rest amplified from 4 to a maximum of 12 DNA bands in the size range 0.5–2.5 kbp (Fig. 2A). The dendrogram from UPGMA analysis (Fig. 2B), based on the 28 pairwise similarity index values from the RAPD analyses of the mitochondrial genomes, revealed that the mtDNAs from the six restorer lines were very different from that of the maintainer line. The mtDNAs from the six restorer lines could be grouped into three clusters; those that restored A1 fully formed one group, those that restored both A1 and A2 cytoplasms formed a separate group while those that partially restored A2 but fully restored A1 formed a third group. Each of these groups were distinct from the other.

RAPD analysis of total DNA

The same 60 primers that were used for mtDNA RAPD were also used for total DNA RAPD. Out of the 60 primers used for amplification, 13 primers failed to

Table 2 Restriction fragment sizes of the different mitochondrial genes in the seven sorghum restorer lines. DNA was digested with *Hin*dIII. Sizes are in kilobasepairs

Genes	CS3541	IS23183	IS1066	IS40575	RS29	ICSV89058	IS3436
atpA	9.5, 7.5, 4.0, 2.5	8.0, 5.8, 4.0	8.0, 5.8, 4.0	8.0, 5.8, 4.0	8.0, 5.8, 4.0	8.0, 5.8, 4.0	8.0, 5.8, 4.0
*atp*6	3.3, 2.2	2.2	2.2	2.2	2.2	2.2	2.2
*atp*9	4.3, 3.6, 3.2	3.6, 3.2	3.6, 3.2	4.9, 3.6, 3.2	3.6, 3.2	3.6, 3.2	3.6, 3.2
cox I	4.5	4.5	4.5	4.5	4.5	4.5	4.5
cox II	3.5	3.5	3.5	3.5	3.5	3.5	3.5
cox III	4.8	4.8	4.8	4.8	4.8	4.8	4.8
*nad*2	8.5	8.5	8.5	8.5	8.5	8.5	8.5
*nad*3	4.6	3.1	3.1	3.1	3.1	3.1	3.1
*rps*12	4.6	3.1	3.1	3.1	3.1	3.1	3.1
*rps*13	12.0	12.0	12.0	12.0	12.0	12.0	12.0
*orf*156	2.5	2.5	2.5	2.5	2.5	2.5	2.5

Table 3 Matrix of the dissimilarity values (1-F, calculated as described in the Materials and methods) for the pairwise comparison of the mtDNA RAPD profiles (lower triangular matrix) and total DNA RAPD profiles (upper triangular matrix)

	Total DNA						
	1	2	3	4	5	6	7
1		0.276	0.292	0.286	0.329	0.314	0.403
2	0.320		0.327	0.313	0.300	0.294	0.395
3	0.361	0.340		0.167	0.284	0.278	0.436
4	0.449	0.426	0.218		0.256	0.242	0.408
5	0.437	0.414	0.319	0.322		0.099	0.405
6	0.414	0.376	0.298	0.281	0.136		0.403
7	0.510	0.525	0.449	0.424	0.462	0.437	
	Mitochondrial DNA						

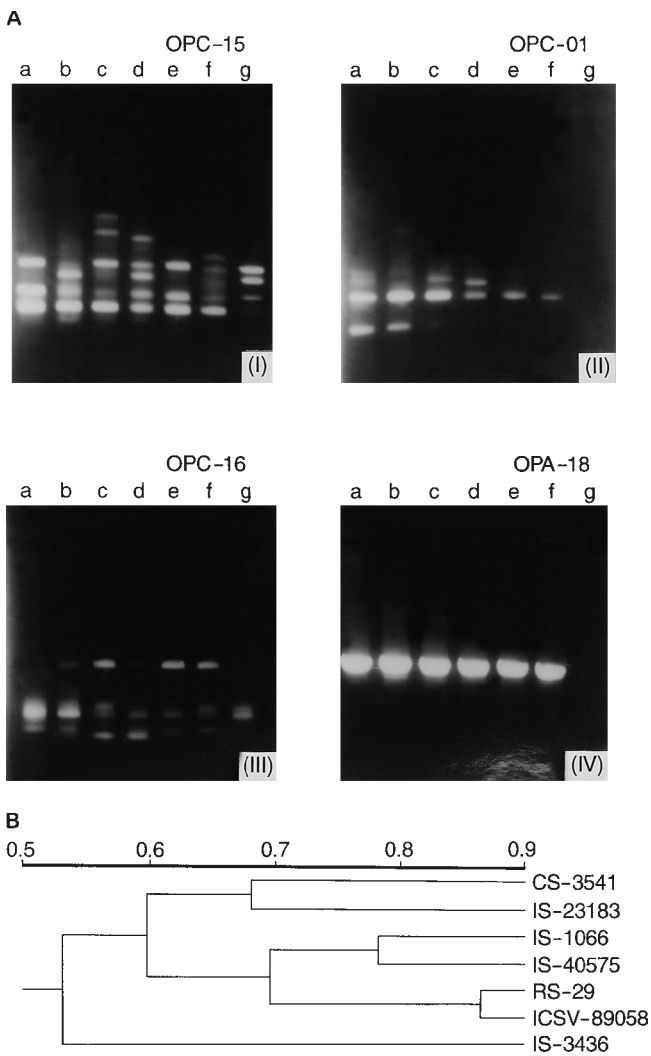

Fig. 2A,B **A** RAPD agarose gel electrophoresis profiles obtained with seven sorghum restorer mtDNAs using primers OPA-18, OPC-01, OPC-15 and OPC-16. *Lanes* marked *a–g* are as serial no 1–7 in Table 1. **B** UPGMA dendrogram generated from the RAPD data of the seven sorghum restorer mtDNAs

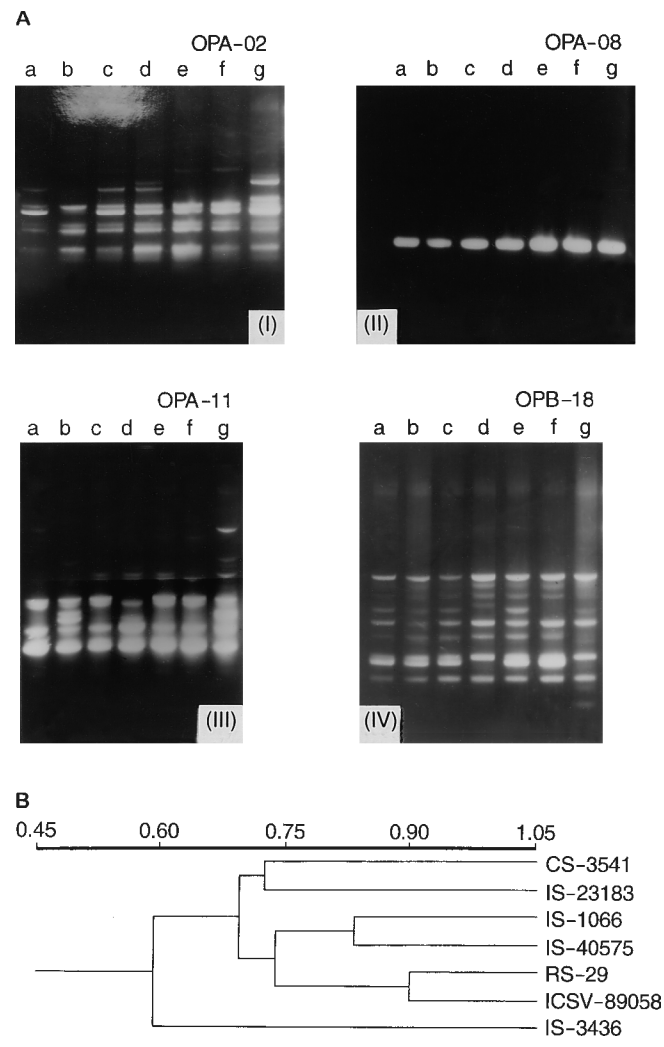

Fig. 3A,B **A** RAPD agarose gel electrophoresis profiles of the seven sorghum total DNAs obtained using the primers OPA-02, OPA-08, OPA-11 and OPB-18. *Lanes* marked *a–g* are as serial no 1–7 in Table 1. **B** UPGMA dendrogram generated from the RAPD data of the seven sorghum restorer total DNAs

amplify, while from amongst the rest, profiles generated by 38 primers were considered for computational analysis since these were consistent. These profiles included a total of 254 DNA bands, of which 65% were polymorphic. The mean dissimilarity value was calculated to be 0.234 for 28 pairwise combinations of seven

genotypes (Table 3). In this case, the primer OPA-8 amplified a single monomorphic band while the others amplified from 5 to a maximum of 11 DNA bands in the range of 0.4–2.5 kbp (Fig. 3A). The dendrogram obtained from the UPGMA analysis (Fig. 3B) based on the pairwise similarity index revealed that the two exclusive A1 restorers pair together. Two of the four A2 restorers, IS1066 and IS40575 (complete A1-A2 restorers), paired together and were placed near the partial A2 restorers RS29 and ICSV89058. However, the maintainer IS3436 was very different from the restorers.

Discussion

The present investigation was undertaken with the aim of characterizing the cytoplasms that are associated with different restorer/maintainer lines in sorghum. Since cytoplasmic male sterility and fertility in plants is a consequence of specific nuclear-mitochondrial interactions, we were interested in finding out if the different restorer lines with different restoration abilities are themselves characterized by specific nuclear-cytoplasmic combinations. A combination of mtDNA RFLP along with RAPD analyses of mtDNA and total DNA was chosen for the purpose. Based on the results, we can make the following important points:

1) that mtDNA RFLP analysis alone is inadequate to characterize the seven cytoplasms, especially with reference to nuclear-mitochondrial interactions;
2) that total DNA RAPD analysis discriminates between the different restorers, such that the clustering pattern based on RAPD similarities clearly conforms to that based on field restoration characteristics;
3) that RAPD analysis of the isolated mtDNAs also results in a clustering of the restorers which fully correlates with their restoration abilities as well as the total DNA RAPD pattern.

Polymorphism in mtDNA was observed with 5 of the 11 RFLP probes used, *atp*A, *nad*3, *rps*12, *atp*6 and *atp*9, such that these mainly differentiated the A1 restorer (CS3541) from the others. Similarly, the A1-A2 restorer, namely, IS40575, could be distinguished from CS3541 and the rest by polymorphism in *atp*9. Other than these, the mtDNA RFLPs failed to distinguish between the different lines. This clearly indicated that the differences, if any, amongst these lines are probably distributed randomly relative to the mtDNA probes used for the RFLPs. The inability of the RFLP approach to distinguish between the various restorer lines is not surprising considering that mitochondrial genes are highly conserved and usually show heterogeneity only at their 5′ and/or 3′ flanking portions (Gray et al. 1992) with the result that this heterogeneity is not always detectable by RFLP probes. It was, therefore, decided to carry out RAPD analyses using isolated mtDNA as templates.

The RAPD technique is simple to carry out, yet it is powerful enough to discriminate between different DNAs. Despite the simplicity and power of the resolution of this technique, it has so far been used in determining mtDNA diversity only in sugar beets (Lorenz et al. 1994, 1997) and more recently by us to distinguish between the different CMS lines in rice (Sane et al. 1997). In view of the limited information generated by mtDNA RFLP, the use of the RAPD technique for determining diversity in the restorer cytoplasms was considered necessary. While RAPD profiles of total DNA would occassionally include bands amplified from plastid and mtDNA (Lorenz et al. 1994), precautions were taken to minimize nuclear and plastid DNA contamination in mtDNA preparations (as described in Materials and methods). Furthermore, by using the isolated mtDNA preparations separately, we were able to identify extra bands in total DNA profiles that may be due to mtDNA present in the total DNA. Likewise, any band present in the mtDNA profile that is also present in the total DNA profile may be due to nuclear DNA contaminating the mtDNA preparation. Such bands can be ignored in the final analysis.

The RAPD data in a majority of the cases did not reveal the presence of these extra bands, indicating thereby that our DNA preparations were reasonably "clean" or that the contamination, if present, was not contributing to the profile. While selecting primer profiles for the computational analysis, we also ignored those few profiles in which such extra bands were present. After this pruning of the data, the profiles that were finally collected included 254 and 120 bands from 38 and 17 primers in the case of total DNA and mtDNA RAPDs, respectively. The UPGMA dendrograms were computed from these data according to Sokal and Sneath (1963).

The validity of the approach can be judged from the results of this study and the data given in Figs. 2B and 3B. The different restorers could clearly be distinguished from each other and this distinction, which was based on nuclear RAPD profiles, paralleled that made on the basis of their restoration characteristics. The lines which restored only the A1 cytoplasm formed one group; the lines which restored both A1 and A2 cytoplasms fully, formed another group while the lines that restored A1 completely but A2 partially, formed a third group. The maintainer line was found to be very different from the restorer lines.

The analysis of the mtDNA RAPD profiles surprisingly also revealed a very similar grouping of these lines. Here too the different cytoplasms grouped together based on their restoration characteristics, while the maintainer cytoplasm was very different from the rest. These analyses indicated that the different restorer/maintainer nuclei are probably associated with specific cytoplasms (which may be considered to be

compatible with each other). The apparent concordance of the total DNA RAPD, mtDNA RAPD and the restoration behaviour of the cytoplasms leads us to the conclusion that the nuclear and cytoplasmic genomes must be compatible with each other to be able to co-exist and, therefore, also to be selected by the breeders or in nature. It has been believed for a long time that fertility restoration by a nuclear restoration gene must result from the functional compatibility of the two genomes, a belief that is strengthened by our results with RAPDs.

The results presented in this communication are important since they demonstrate the potential of the cytoplasms to be used as possible markers in the selection of restorer or maintainer lines. Our investigation also demonstrates the advantage of the RAPD technique in characterizing the diversity in the cytoplasms of crop plants. The technique could thus find tremendous use in the study of CMS and fertility restoration, both for a detailed molecular study as well as for a survey of cytoplasmic diversity for subsequent exploitation in commercial hybrid seed production.

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