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Molecular diversity of male sterility inducing and male-fertile cytoplasms in the genus *Helianthus*

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Abstract The organisation of mtDNA was investigated for 28 sources of cytoplasmic male sterility (CMS) and a fertile line (normal cytoplasm) of *Helianthus annuus* by Southern hybridisation. In addition to nine known mitochondrial genes (*atp6*, *atp9*, *cob*, *coxI*, *coxII*, *coxIII*, *18S*, *5S* and *nd5*) three probes for the open reading frames in the rearranged area of PET1, *orfH522*, *orfH708* and *orfH873*, were used. Genetic similarities of the investigated cytoplasms varied between 0.3 and 1. Cluster analyses using the UPGMA method allowed the distinction of ten mitochondrial (mt) types between the 29 investigated cytoplasms. Most mitochondrial types comprise two or more CMS sources, which could not be further separated, like the PET1-like CMS sources (with the exception of ANO1 and PRR1), or ANN1/ANN2/ANN3, ANN4/ANN5, ARG3/RIG1, BOL1/EXI1/PEF1/PEP1 and GIG1/PET2. ANL1, ANL2 and the fertile cytoplasms are also regarded as one mitochondrial type. Unique banding patterns were only observed for ANT1 (*atp6*), MAX1 (*atp6*, *orfH522* and *orfH708*) and PRR1 (*coxII*). However, four of the mitochondrial types showed unique hybridisation signals: ANN4/ANN5 had characteristic bands for *atp6* and *orfH708*, PEF1/PEP1/EXI1/BOL1 for *atp6* and *coxII*, and PET2/GIG1 for *atp9*. The PET1-like cytoplasms all shared the same patterns for *orfH522*, *orfH708* and *cob* (except ANO1). It could be demonstrated that CMS sources, like, e.g., PET2 and PEF1, are different from PET1 in mtDNA organisation and the CMS mechanism. Therefore, these CMS sources represent interesting candidates for the development of new hybrid breeding systems based on new CMS mechanisms.

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Introduction

Hybrid breeding in sunflower is based on a single source of cytoplasmic male sterility (CMS), the so-called PET1 or French cytoplasm, which originates from an interspecific cross between *Helianthus petiolaris* and *Helianthus annuus* (Leclercq 1969). However, the use of the male-sterile T cytoplasm in maize clearly demonstrated the high risk involved in using only one cytoplasm for commercial hybrid breeding, as the fungus *Helminthosporium maydis* specialized for the T cytoplasm resulted, in heavy yield losses and the total omittance of the T cytoplasm for further hybrid breeding (Ullstrup 1972). In sunflower, more than 62 CMS sources are now available (Serieys 1999) and can be used for hybrid breeding. However, to build up new restorer and maintainer pools is cost and time intensive. Therefore, thorough investigations of the available CMS cytoplasms on the molecular level are advisable to identify cytoplasms different in respect of the CMS mechanism and the organisation of the mitochondrial DNA.

In PET1, a new open reading frame *orfH522* in the 3′-flanking region of the *atpA* gene could be associated with the CMS phenotype (Köhler et al. 1991; Laver et al. 1991). Using specific antibodies against the gene product of *orfH522* it was demonstrated that *orfH522* encodes the 16-kDa protein (Monéger et al. 1994; Horn et al. 1996), which represents the unique difference between the in organello translation products of fertile and male-sterile lines (Horn et al. 1991). Comparing the mitochondrially encoded proteins of 28 CMS sources in sunflower, nine additional CMS sources could be identified that also have the same CMS mechanism as PET1 (Horn et al. 1996). This was surprising as these PET1-like CMS sources had different origins (Horn and Friedt 1999). According to Serieys (1996) these cytoplasmic male-sterile germplasms had been produced by either different interspe-

cific crosses involving *Helianthus argophyllus* (ARG1), *Helianthus neglectus* (NEG1), *Helianthus exilis* (EXI2) and *Helianthus anomalus* (ANO1), and two subspecies of *Helianthus praecox* (PRR1, PRH1), or by mutagenesis of two maintainer lines for the PET1 cytoplasm (MUT1 and MUT2). In addition, one of the CMS types that arose spontaneously (ANN10) expressed the 16-kDa protein. All these PET1-like CMS cytoplasms showed the same organization at the *atpA* locus (Horn and Friedt 1999). Apart from these PET1-like CMS sources, other groups of CMS cytoplasms could be identified which expressed new proteins (Horn and Friedt 1999). ARG3 and RIG1 showed an additional 16.9-kDa protein but missed a 17.5-kDa protein common to the other cytoplasms. ANN1 and ANN3 expressed three specific proteins of 34.0, 16.9 and 16.3 kDa in common. A protein of 12.4 kDa was unique for PET2 and GIG1.

I have now investigated the mitochondrial organization of these 28 CMS sources and the fertile cytoplasm by Southern hybridisation using probes distributed all over the mitochondrial genome in sunflower (Siculella and Palmer 1988) and estimated their genetic similarities. Cluster analyses employing the UPGMA method made it possible to group the cytoplasms according to the organization of the mitochondrial DNA. The necessity of molecular investigations of CMS sources prior to the use of commercial hybrid breeding is discussed.

Materials and methods

CMS sources

Apart from the fertile cytoplasm of *H. annuus* 28 CMS sources different by their origin were investigated at the mtDNA level. The origin of the CMS sources, which were kindly provided by Dr. Hervé Serieys (Montpellier, INRA), is given in Table 1.

Probes used for Southern hybridisation

Probes for *coxII* and *atp9* were amplified by PCR using the sequence-specific primers *coxII*for (5′-CGAGAAATAGATGCTCAGCCTG-3′)/*coxII*rev (5′-GATAATGCGCAGTGGAAAGG-3′) and *atp9*for (5′-GGTGCAAAATCAATAGGGGCCG-3′)/*atp9*rev (5′-ACCGAATGAATGCGTCACAAGG-3′) that have been derived from the gene bank accessions given in Table 2. PCR amplification was performed in a Perkin Elmer 9600 thermocycler using 50 ng of DNA (RHA325) with an initial denaturing step of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 53°C and 30 s at 72°C and a final extension step of 7 min at 72°C, followed by a hold at 8°C. In addition to the PCR products, cloned restriction fragments of mitochondrial genes from sunflower and *Oenothera* (Table. 2) were used for Southern hybridisations. PCR products and restriction fragments were run at 120 V on 0.8% low-melt agarose gels, corresponding bands were excised after

Table 1 Overview of the investigated CMS sources (References are cited in Serieys 1996)

Name	FAO-code	Origin	Reference
1. Spontaneously occurring CMS sources			
H. annuus 367	ANN1	*H. annuus*	Serieys and Vincourt 1987
H. annuus 517	ANN2	*H. annuus*	Serieys and Vincourt 1987
H. annuus 519	ANN3	*H. annuus*	Serieys and Vincourt 1987
H. annuus 521	ANN4	*H. annuus*	Serieys and Vincourt 1987
NS-ANN-81	ANN5	*H. annuus*	Skoric 1988
AN-67	ANN10	*H. annuus*	Christov 1992
2. Intraspecific crosses			
Kouban	ANL1	*H. annuus* ssp. *lenticularis*	Anashchenko et al. 1974
Indiana1	ANL2	*H. annuus* ssp. *lenticularis*	Heiser 1982
Fundulea 1	ANT1	*H. annuus* ssp. *texanus*	Vranceanu et al. 1986
3. Interspecific crosses			
Anomalus	ANO1	*H. anomalus*	Serieys and Vincourt 1987
Argophyllus	ARG1	*H. argophyllus*	Christov 1990
Argophyllus	ARG2	*H. argophyllus*	Christov 1990
Argophyllus	ARG3	*H. argophyllus*	Christov 1992
Bolanderi	BOL1	*H. bolanderi*	Serieys and Vincourt 1987
Exilis	EXI1	*H. exilis*	Serieys and Vincourt 1987
Exilis	EXI2	*H. exilis*	Serieys 1991
CMG2	GIG1	*H. giganteus*	Whelan 1981
CMG3	MAX1	*H. maximiliani*	Whelan and Dedio 1980
Neglectus	NEG1	*H. neglectus*	Serieys and Vincourt 1987
Fallax	PEF1	*H. petiolaris* ssp. *fallax*	Serieys and Vincourt 1987
PET/PET	PEP1	*H. petiolaris* ssp. *petiolaris*	Serieys and Vincourt 1987
Petiolaris	PET1	*H. petiolaris*	Leclercq 1969
CMG1	PET2	*H. petiolaris*	Whelan and Dedio 1980
PHIR 27	PRH1	*H. praecox* ssp. *hirsutus*	Christov 1993
PRUN 29	PRR1	*H. praecox* ssp. *runyonii*	Christov 1993
Vulpe	RIG1	*H. rigidus*	Vulpe 1972
4. Induced by mutagenesis			
HEMUS	MUT1	Irradiation of ‘Hemus’	Christov 1993
PEREDOVIK	MUT2	Sonification ‘Peredovik’	Christov 1993

Table 2 Mitochondrial probes used for Southern hybridisations

Gene	Translation product	Restriction site	Size	Reference/accession
atpA	F_1-ATPase, α-subunit	*Eco*RI/*Sac*I	1.0 kb	Köhler et al. 1991
atp6	F_0-ATPase, subunit 6	*Nhe*I	1.5 kb	Schuster and Brennicke 1987
atp9	F_0-ATPase, subunit 9	–	474 bp	Accession X51895
cob	Apocytochrom b	*Eco*RI	1.5 kb	Köhler et al. 1991
coxI	Cytochromoxidase, subunit I	*Eco*RI	1.95 kb	Hiesel et al. 1987
coxII	Cytochromoxidase, subunit II	–	764 bp	Accession X62341
coxIII	Cytochromoxidase, subunit III	*Eco*RI/*Pst*I	1.1 kb	Hiesel et al. 1987
orfH522	16-kDa protein	*Taq*I	0.4 kb	Köhler et al. 1991
18S-rRNA	–	*BamH*I	5.2 kb	Brennicke et al. 1985
5S-rRNA	–	*BamH*I	5.2 kb	Brennicke et al. 1985
nd5	NADH-dehydrogenase, subunit 5	*BamH*I	5.2 kb	Brennicke et al. 1985
orfH873	Unknown	*SmaI/EcoRI*	400 bp	Köhler et al. 1991
orfH708	Unknown	*Sma*I/*Hind*II	339 bp	Köhler et al. 1991

staining with ethidium bromide, diluted with 10 µl of TE and heated for 10 min at 96°C.

Southern hybridisation against mitochondrial DNA

Mitochondria were isolated from etiolated sunflower seedlings by differential centrifugation as described by Horn et al. (1991). However, the mitochondria were not further purified by the Percoll density gradient but were immediately treated with DNase and then centrifuged for 10 min at 10,000 rpm, 8°C. The sediments were dissolved in 1 ml of lysis buffer [50 mM Tris-HCl pH=7.2, 5 mM ethylene diamine tetracetic acid (EDTA)] and frozen at –80°C. Isolation of the mitochondrial DNA was performed according to Rogers and Bendich (1985) using cetyltrimethylammoniumbromide (CTAB). The mtDNA was digested with different restriction enzymes according to the manufacturer's manuals and separated on 0.8% agarose gels. The probes used for Southern hybridisation are given in Table 2.

The probes were labelled non-radioactively by the enhanced chemiluminescence (ECL) system according to the Amersham Life Science protocol. Hybridisation was performed over night at 42°C, followed by two washes of 20 min at 42°C in Wash I [6 M urea, 0.4% sodiumdodecylsulfate (SDS), 0.5× SSC] and another wash step for 5 min at 42°C in Wash II (2× SSC). The filters were incubated in the detection solution before exposure to a film. The filters were re-hybridised with new probes under the same conditions.

Analysis of the RFLP profiles

Genetic similarities (Sn) were estimated according to the formula developed by Nei and Li (1979): $Sn_{xy}=2\ n_{xy}/n_x+n_y$, ($n_{xy}$ represents the number of bands shared by genotype X and Y; n_x and n_y are the total number of bands shown by genotype X and Y, respectively). Applying the programme NTSYS-pc (Rohlf 1993) cluster analyses based on the matrix of genetic similarity were performed using the unweighted pair group method of arithmetic means (UPGMA). The results of the cluster analyses are shown as a dendrogram. A matrix of cophenetic values based on the 'tree matrix' was produced by the programme NTSYS-pc. The Mantel matrix correspondence test (Mantel 1967) was used to compare the cophenetic matrix with the similarity matrix to define the degree of congruence in the estimation of genetic relationships.

Results

Southern hybridisation with different mitochondrial probes

Comparison of the mitochondrial organisation of 28 CMS sources and the fertile cytoplasm in *Helianthus* was performed by Southern hybridisation of mtDNA digested with *Bgl*II against nine known mitochondrial genes (*atp6*, *atp9*, *cob*, *coxI*, *coxII*, *coxIII*, and *18S*, *5S* and *nd5*). In addition, the three identified open reading frames of the PET1 cytoplasm, *orfH522*, *orfH708* and *orfH873* (Köhler et al. 1991), were used as probes. The banding patterns and the frequencies of fragments are given in Table 3.

There were only very few unique banding patterns produced by Southern hybridisation. MAX1 is the only CMS source that apart from the PET1-like CMS sources gave a signal with *orfH522*. However, the signal size with 10.2 kb is unique for MAX1, as well as the 3.0-kb signal obtained for this CMS source using *atp6* as a probe. In addition, MAX1 showed a unique fragment of 2.4 kb with *orfH708*. All three polymorphisms are potential candidates for molecular changes involved in creating the CMS phenotype in MAX1. In addition, ANT1 demonstrated a unique fragment of 8.1 kb in the hybridisation experiments with *atp6*. PRR1, one of the PET1-like cytoplasms, showed a characteristic additional fragment of 4.0 kb with *coxII* as a probe.

However, there are groups of cytoplasms that shared hybridisation signals. All PET1-like cytoplasms showed a fragment of 4.9 kb with *orfH522* as a probe, a fragment of 1.2 kb with *orfH708* and a fragment of 8.9 kb with *cob* (except ANO1). ANN4/ANN5 shared a hybridisation signal of 6.2 kb with *atp6* and of 1.4 kb with *orfH708*, not detected in any of the other investigated cytoplasms. The CMS sources PET2/GIG1 had an additional unique band of 4.7 kb with *atp9*, apart from the fragment of 10.3 kb also present in 25 other cytoplasms in sunflower. Also the group PEF1/PEP1/EXI1/BOL1 showed a characteristic band of 5.0 kb with *atp6* and of 7.8 kb with *coxII*.

Using *atp6* as a probe allowed the highest degree of differentiation between the cytoplasms (Fig. 1). Seven different hybridisation patterns were obtained against *Bgl*II-digested mtDNA. Fragment sizes of 3.0 kb, 5.0 kb, 5.9 kb, 6.2 kb, 8.1 kb and 9.3 kb were observed (Fig. 1). PET2/GIG1 (5.9 kb and 9.3 kb) and MAX1 (3.0 kb and 9.3 kb) showed two signals, as well as ANN4 and ANN5 (6.2 and 9.3 kb). For all other cytoplasms only one signal was obtained.

Table 3 Hybridisation patterns of the mtDNA of 28 CMS sources and the fertile cytoplasm of *H. annuus* digested with *Bgl*II against known mitochondrial genes and open reading frames. Different mitochondrial types are shown in different colours. Unique hybridisation signals for mitochondrial types have a yellow background for groups and a red colour for single cytoplasms

Probe	kb	ARG2	ANN4	ANN5	GIG1	PET2	ARG3	RIG1	ANL1	ANL2	ORF79	BOL1	EXI1	PEF1	PEP1	MAX1	ANN10	ARG1	EXI2	MUT1	MUT2	NEG1	PET1	PRH1	PRR1	ANO1	ANT1	ANN1	ANN2	ANN3	Frequency
atp6	9.3	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	9
	8.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
	6.2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	5.9	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	17
	5.0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
atp9	11.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	10.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	25
	7.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	4.7	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
cob	10.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	8.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	9
	8.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	6.3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	15
	3.4	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
	2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	1.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29
	0.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29
coxI	11.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	10.7	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	22
	10.1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
coxII	7.8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	5.1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25
	4.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
	2.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29
coxIII	8.7	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11
	6.6	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	15
	5.3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	5
18S/5S/ nd5	11.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	4
	10.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	25
	8.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	29
orfH522	10.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	4.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	10
orfH708	6.2	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
	3.4	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	1.4	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	10
orfH873	9.9	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	6
	8.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	12
	8.5	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	11

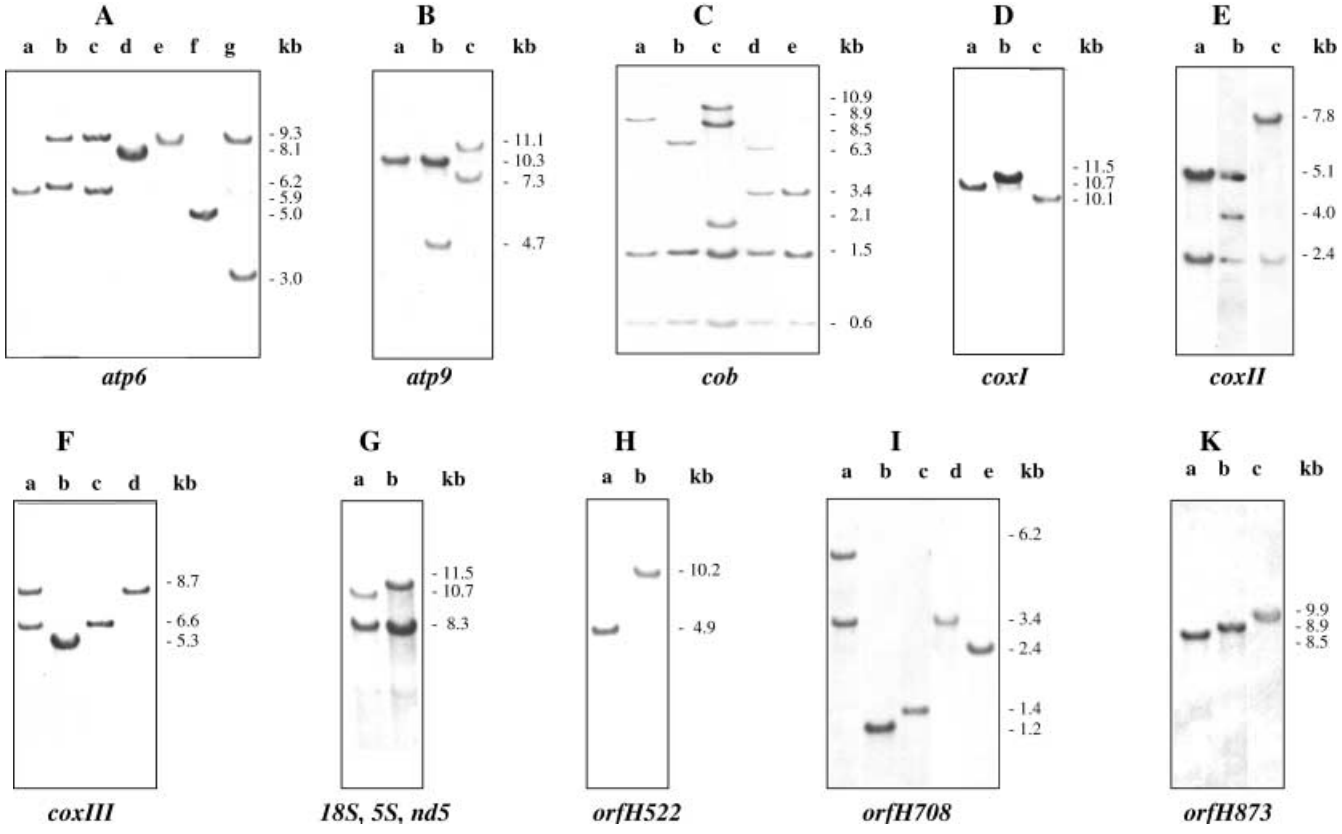

Fig. 1A–K Typical patterns of Southern hybridisation against mitochondrial DNA of 28 CMS sources and the fertile cytoplasm of *H. annuus* using *atp6*, *atp9*, *cob*, *coxI*, *coxII*, *coxIII*, and *18S*, *5S* and *nd5*, as well as *orfH522*, *orfH708* and *orfH873* as probes. The mitochondrial DNA was digested with *BglII*. Typical patterns for a probe are examplified by one of the cytoplasms: **A** *atp6* (*a* PET1, *b* ANN4, *c* PET2, *d* ANT1, *e* ARG2, *f* PEP1, *g* MAX1); B. *atp9* (*a* Fertile, *b* GIG1, *c* ANN1); **C** *cob* (*a* PET1, *b* PET2, *c* ANT1, *d* ANL2, *e* ARG2); **D** *cox*I (*a* MAX1, *b* ANT1, *c* Fertile), **E** *coxII* (*a* ANN3, *b* PRR1, *c* GIG1); **F** *cox*III (*a* ANL2, *b* ANT1, *c* ANN5, *d* PET1); **G** *18S*, *5S*, *nd5* (*a* MAX1, *b* ANT1); **H** *orfH522* (*a* PET1, *b* MAX1); **I** *orfH708* (*a* ANL2, *b* PET1, *c* ANN5, *d* ARG2, *e* MAX1); **K** *orfH873* (*a* Fertile, *b* PET1, *c* BOL1)

Hybridisation with *atp9* resulted in three different patterns. Twenty-five of the cytoplasms showed one signal of 10.3 kb. For PET2 and GIG1 an additional signal of 4.7 kb was observed, and for ANT1, ANN1, ANN2 and ANN3 two different fragments of 7.3 kb and 11.1 kb hybridised with *atp9* as a probe.

For *cob* as a probe five different hybridisation patterns were obtained. However, all cytoplasms shared two fragments of 0.6 kb and 1.5 kb. The fertile cytoplasm and ANL2 showed two additional bands of 3.4 kb and 6.3 kb. ANT1, ANN1, ANN2 and ANN3 had three additional bands of 2.1 kb, 8.5 kb and 10.9 kb. All other cytoplasms had only one additional band: ARG2 of 3.4 kb, the PET1-like cytoplasms except ANO1 of 8.9 kb and the remaining cytoplasms of 6.3 kb.

Hybridisation with *coxI* as a probe resulted in three patterns of a single signal (Fig. 1). For most of the cytoplasms this band had a size of 10.7 kb. The CMS sources ANN1, ANN2, ANN3 and ANT1 showed a signal of 11.5 kb, whereas RIG1, ARG2 and ARG3 gave a signal with a fragment of only 10.1 kb.

For *coxII*, all cytoplasms apart from PRR1 showed two hybridisation signals, one of 2.4 kb shared by all cytoplasms and one of 5.1 kb present in all apart from BOL1, EXI1, PEF1 and PEP1 which instead had a signal of 7.8 kb. PRR1 is the only cytoplasm for which three signals of 2.4 kb, 4.0 and 5.1 kb were detected.

Using *coxIII* as a probe four different patterns were observed. Nearly all CMS sources showed one signal that had a size either of 5.3 kb, 6.6 kb or 8.7 kb (Fig. 1). In the fertile cytoplasm and ANL2, two signals of 6.6 kb and 8.7 kb were observed. With regard to the PET1-like CMS sources, *coxIII* was able to differentiate ANO1 from the other PET1-like CMS sources. ANO1 showed a fragment of 6.6 kb whereas all other CMS sources of this group showed an 8.7-kb fragment.

Hybridisation analyses with a probe containing *18S*, *5S* and *nd5* resulted in two signals for all cytoplasms (Fig. 1). A fragment of 8.3 kb was shared by all cytoplasms. For the second signal most cytoplasms showed a 10.7-kb fragment and only ANN1, ANN2, ANN3 and ANT1 gave a larger signal of 11.5 kb so that two different patterns were detected (Table 3).

Of the three open reading frames present in the rearranged area of PET1 only *orfH873* gave hybridisation signals in all 29 investigated cytoplasms (Table 3). Three patterns of single bands with sizes of 8.5 kb, 8.9 kb and 9.9 kb were observed (Fig. 1). ANN1, ANN2, ANN3

Table 4 Similarity matrix calculated according to Nei and Li (1979) for male sterility inducing and fertile cytoplasms in the genus *Helianthus*

	ARG2	ANT1	ANN1	ANN2	ANN3	BOL1	EXI1	PEF1	PEP1	MAX1	ANN4	ANN5	ARG3	RIG1	ANN10	ANO1	ARG1	EXI2	MUT1	MUT2	NEG1	PET1	PRH1	PRR1	GIG1	PET2	ANL1	Fertile	ANL2
ARG2	1.000																												
ANT1	0.500	1.000																											
ANN1	0.500	0.867	1.000																										
ANN2	0.500	0.867	1.000	1.000																									
ANN3	0.500	0.867	1.000	1.000	1.000																								
BOL1	0.480	0.296	0.296	0.296	0.296	1.000																							
EXI1	0.480	0.296	0.296	0.296	0.296	1.000	1.000																						
PEF1	0.480	0.296	0.296	0.296	0.296	1.000	1.000	1.000																					
PEP1	0.480	0.296	0.296	0.296	0.296	1.000	1.000	1.000	1.000																				
MAX1	0.571	0.333	0.400	0.400	0.400	0.741	0.741	0.741	0.741	1.000																			
ANN4	0.667	0.414	0.414	0.414	0.414	0.692	0.692	0.692	0.692	0.759	1.000																		
ANN5	0.667	0.414	0.414	0.414	0.414	0.692	0.692	0.692	0.692	0.759	1.000	1.000																	
ARG3	0.720	0.444	0.370	0.370	0.370	0.667	0.667	0.667	0.667	0.667	0.769	0.769	1.000																
RIG1	0.720	0.444	0.370	0.370	0.370	0.667	0.667	0.667	0.667	0.667	0.769	0.769	1.000	1.000															
ANN10	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000														
ANO1	0.519	0.345	0.345	0.345	0.345	0.769	0.769	0.769	0.769	0.759	0.714	0.714	0.769	0.769	0.786	1.000													
ARG1	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000												
EXI2	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000											
MUT1	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000	1.000										
MUT2	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000	1.000	1.000									
NEG1	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000	1.000	1.000	1.000								
PET1	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000	1.000	1.000	1.000	1.000							
PRH1	0.519	0.345	0.414	0.414	0.414	0.539	0.539	0.539	0.539	0.552	0.571	0.571	0.615	0.615	1.000	0.786	1.000	1.000	1.000	1.000	1.000	1.000	1.0000						
PRR1	0.500	0.333	0.400	0.400	0.400	0.519	0.519	0.519	0.519	0.533	0.552	0.552	0.593	0.593	0.966	0.759	0.966	0.966	0.966	0.966	0.966	0.966	0.966	1.00					
GIG1	0.667	0.414	0.414	0.414	0.414	0.692	0.692	0.692	0.692	0.759	0.857	0.857	0.846	0.846	0.643	0.786	0.643	0.643	0.643	0.643	0.643	0.643	0.643	0.621	1.000				
PET2	0.667	0.414	0.414	0.414	0.414	0.692	0.692	0.692	0.692	0.759	0.857	0.857	0.846	0.846	0.643	0.786	0.643	0.643	0.643	0.643	0.643	0.643	0.643	0.621	1.000	1.000			
ANL1	0.667	0.414	0.345	0.345	0.345	0.692	0.692	0.692	0.692	0.690	0.786	0.786	0.846	0.846	0.643	0.786	0.643	0.643	0.643	0.643	0.643	0.643	0.643	0.621	0.857	0.857	1.000		
Fertile	0.690	0.387	0.323	0.323	0.323	0.643	0.643	0.643	0.643	0.645	0.733	0.733	0.786	0.786	0.667	0.733	0.667	0.667	0.667	0.667	0.667	0.667	0.667	0.645	0.800	0.800	0.933	1.000	
ANL2	0.690	0.387	0.323	0.323	0.323	0.643	0.643	0.643	0.643	0.645	0.733	0.733	0.786	0.786	0.667	0.733	0.667	0.667	0.667	0.667	0.667	0.667	0.667	0.645	0.800	0.800	0.933	1.000	1.000

and the PET1-like CMS cytoplasms (except ANO1) showed a band of 8.9 kb. PEF1, PEP1, EXI1, ANO1, MAX1 and BOL1 gave a signal at 9.9 kb, and all other cytoplasms showed a band at 8.5 kb. Investigating the 29 cytoplasms, hybridisations obtained with *orfH708* were highly polymorphic and signals were only detected in the fertile cytoplasm as well as in ANN4, ANN5, ANL1, ANL2, ARG2, the PET1-like cytoplasms and MAX1. Hybridisation signals with *orfH522* were only obtained for the PET1-like CMS sources and MAX1.

Genetic similarity between 29 cytoplasms in sunflower

The genetic similarity of the mitochondrial DNA of the investigated CMS sources and the fertile (normal) cytoplasm, which was estimated according to Nei and Li (1979), varied between 0.3 and 1 (Table 4). The genetic similarity between the PET1-like CMS sources, with the exception of ANO1 and PRR1 is 1, and 0.67 for the fertile cytoplasm. Between ANO1, the PET1-like CMS-sources (except PRR1) and the fertile cytoplasm, the genetic similarity is 0.79 and 0.73, respectively (Table 4). The genetic similarity between PRR1 and the PET1-like sources (except ANO1) is 0.97.

The genetic similarity between the CMS sources, which occurred spontaneously in *H. annuus* (Table 1), varied between 0.41 and 1. CMS sources such as ANN1, ANN2 and ANN3 were indistinguishable from each other as well as ANN4 and ANN5. However, eight out of ten probes differentiated these two groups from each other. Therefore, the genetic similarity was only 0.41 between these two groups. Of the spontaneously occurring CMS sources, ANN10 showed the highest similarity of 0.67 to the fertile cytoplasm of *H. annuus*.

ANL1 and ANL2 have a genetic similarity of 0.8 and 1 to the fertile cytoplasm, respectively. However, ANT1 originating from an intraspecific cross with the subspecies *H. annuus texanus* only showed a similarity of 0.39 to the fertile cytoplasm. The CMS sources induced by mutagenesis (MUT1 and MUT2) in *H. annuus* had a similarity of 0.67 to the fertile cytoplasm.

In the group of CMS sources that were obtained by interspecific hybridisation with *H. petiolaris* (PET1 and PET2) and the subspecies *H. petiolaris fallax* (PEF1), and *H. petiolaris petiolaris* (PEP1), the genetic similarity varied between 0.54 and 1. For those CMS sources (ARG1, ARG2 and ARG3) that were developed from interspecific crosses with *H. argophyllus*, values between 0.52 and 0.72 were estimated. Between CMS sources (GIG1, MAX1 and RIG1) originating from the section *Divaricati* the similarity varied between 0.67 and 0.85. Comparing GIG1, MAX1 and RIG1 to the fertile cytoplasm, values between 0.65 and 0.8 were observed.

Cluster analyses were based on the similarity matrix (Table 4), using the UPGMA method, and genetic relationships between the CMS sources were visualized as a dendrogram (Fig. 2). These UPGMA-cluster analyses

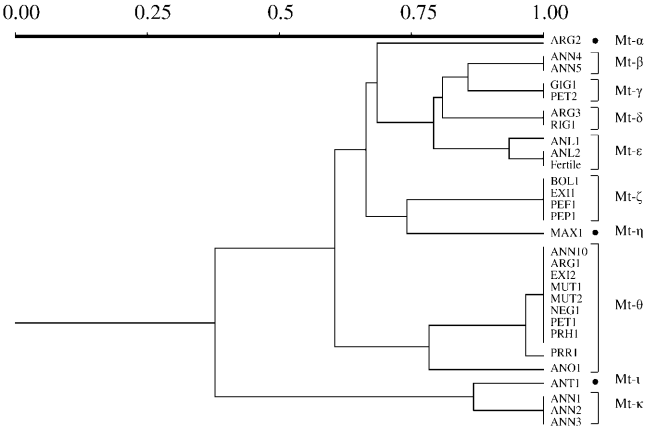

Fig. 2 Dendrogram of 28 male sterility inducing and one fertile cytoplasm in the genus *Helianthus* generated by RFLP data using the UPGMA method

allowed to differentiate ten mitochondrial types between the 29 cytoplasms (one fertile and 28 CMS sources). Most mitochondrial types comprise two or more CMS sources, which could not be further separated, like the PET1-like CMS sources (with exception of ANO1 and PRR1), or ANN1/ANN2/ANN3, ANN4/ANN5, ARG3/RIG1, BOL1/EXI1/PEF1/PEP1, and GIG1/PET2. ANT1, MAX1 and ARG2 so far represent unique mitochondrial types. ANL1, ANL2 and the fertile cytoplasm are regarded as one mitochondrial type. Mitochondrial types were classified as mt-α to mt-κ following the UPGMA dendrogram, as it is too preliminary to name a characteristic cytoplasm for each group as for the PET1-like cytoplasms that represent the type mt-θ.

Comparing the cophenetic matrix with the similarity matrix by the Mantel matrix the correspondence test resulted in a cophenetic correlation coefficient of r=0.96, confirming the efficiency of the UPGMA method in estimating genetic relationships as the degree of distortion in relation to the genetic similarity is minimal.

Detailed molecular analyses of the PET1-like cytoplasms

The PET1-like cytoplasms are the largest group of cytoplasms that have a different origin, but could not be differentiated by Southern analysis with the exception of ANO1 and PRR1. Therefore, these cytoplasms were further investigated by hybridisations with the same probes but against mtDNA digested with different restriction enzymes: *coxI* (*Asp*718, *Sma*I), *coxIII* (*Asp*718, *Sal*I), *orfH522* (*Sal*I, *Asp*718, *Dra*I, *Hind*III), *18 S/5S* and *nd5* (*Bam*HI), *atp6* (*Bam*HI), *orfH708* (*Sal*I) and *cob* (*Sal*I). In addition, *atpA* (*Bgl*II) was applied as a probe. However, by using these 13 additional probe/enzyme combinations (data not shown) no further differentiation between the PET1-like cytoplasms (except ANO1 and PRR1) was possible.

Discussion

Estimation of the genetic similarity and cluster analyses using the UPGMA method, which were based on the RFLP data, demonstrated that groups of cytoplasms are very similar in the organisation of the mitochondrial DNA because most mitochondrial types comprise two or more CMS sources, like the PET1-like CMS sources (except ANO1 and PRR1) or ANN1/ANN2/ANN3, ANN4/ANN5, ARG3/RIG1, BOL1/EXI1/PEF1/PEP1 and GIG1/PET2, that could not be further distinguished. Most of the mitochondrial types were characterized by unique banding patterns. However, ANN1/ANN2/ANN3 shared characteristic bands for *atp9*, *cob*, *coxI* and *18S*, *5S* and *nd5* with ANT1 which, due to additional differences, formed a separate cluster.

The results obtained by Southern hybridisation are supported by the analyses of the expressed mitochondrial proteins of these cytoplasms (Horn et al. 1996; Horn and Friedt 1999). The same groups can be formed according to the patterns of the mitochondrially encoded proteins. However, no specific proteins were observed for BOL1/EXI1/PEF1/PEP1 and ANN4/ANN5.

Crouzillat et al. (1991, 1994) investigated 16 cytoplasms (15 male-sterile and one fertile) in sunflower using 12 mitochondrial probes and could differentiate 13 of the cytoplasms, but were also not able to distinguish the groups ANN1/ANN2/ANN3 and PEF1/PEP1. However, 11 of the 12 probes detected polymorphisms by comparing ANN1/ANN2/ANN3 with ANN4. PET2 and GIG1 were polymorphic in hybridisations with the mitochondrial probes *coxII*, *atpA* and *atp9* (Crouzillat et al. 1991) indicating that these cytoplasms have a very similar organisation but are not identical. However, organisations of the mitochondrial genomes do not seem to be random and certain configurations seem to occur preferentially, especially after the selection for stable male sterility.

The UPGMA dendrogram based on the Southern-hybridisation data is neither in accordance with the phylogeny of the species in the genus *Helianthus* (Rieseberg 1991) nor with the origin of the CMS sources from different interspecific crosses (Serieys 1996, 1999). In their study of 15 CMS sources and the fertile cytoplasm Crouzillat et al. (1994) also came to the conclusion that no correlation between the species and mtDNA-type could be observed. The high degree of introgressions observed within the genus *Helianthus* might be one reason for the lack of correspondence of the CMS sources with their phylogeny or their origin from different interspecific crosses (Rieseberg et al. 1991). In addition, the CMS cytoplasms were all selected by the plant breeders for stable male-sterile types, which might exclude other organisations of the mitochondrial DNA present in the wild species. In sunflower, it is still unclear whether the configurations of the mtDNA observed in the CMS cytoplasms were already present in the wild species or whether these have been newly created by interspecific hybridisation or other events. Rieseberg et al. (1994) investigated 55 accessions of *H. annuus* and 26 accessions of *H. petiolaris* by PCR using PET1-specific primers but could not find any configurations in these accessions corresponding to PET1. Characteristic fragments of MAX1 were detected in one wild accession MAX30 (Hahn and Friedt 1994). However, investigations involving CMS-specific regions of other species indicate a frequent occurrence in the wild relatives. The male sterility inducing *pvs-orf239*-sequence of common bean is widespread in wild populations of *Phaseolus coccineus* and *Phaseolus vulgaris* (Hervieu et al. 1993). A wide distribution of the Ogura-type cytoplasm was found among Japanese wild radishes (*Raphanus sativus*) and Asian radish cultivars (Yamagishi and Terachi 1996), as well as in *Raphanus raphanistrum* (Yamagishi and Terachi 1997).

Investigations on fertility restoration are reported for a number of the CMS cytoplasms included in our study of the organisation of the mtDNA and protein patterns, which support the molecular data. PET2 and GIG1 are restored by RHA294 and partially restored by RPET2 and RGIG1, but maintained by RHA274, RMAX1, RHA265, RHA80, HA89 and P21 (Havekes et al. 1991). These field trials on fertility restoration demonstrate that these two cytoplasms show the same behaviour in test crosses and could be differentiated from PET1, ANL1, ARG1, RIG1, ANN2 and ANN3. Test crosses by Horn and Friedt (1997) using restorer lines for PET1 showed that restorer genes of PET2 and PEF1 are distinct from each other since restorer lines for PEF1 are maintainers for PET2 and vice versa. In addition, ANL1, ANL2, MAX1, ANN1, ANN2, ANN3 and ANN4 could be differentiated from these CMS cytoplasms. ANN4 also showed a different maintainer/restorer pattern than ANN1, ANN2 and ANN3.

The molecular investigations on 28 CMS sources and the fertile cytoplasm in sunflower demonstrate that cytoplasms different by their origin can show a considerable similarity which cannot be expected from their pedigree. Therefore, in sunflower, and probably also in other species like rice (Hoan et al. 1997) or onions (Havey 2000), using a new CMS cytoplasm for hybrid breeding should carefully consider molecular characterisation of the CMS cytoplasms, even if these cytoplasms have a different origin, in order to avoid using the same or a very similar mitochondrial type. In sunflower, PET2 and PEF1 have a clearly different organisation of the mitochondrial DNA from the PET1-like cytoplasms and represent interesting new sources of male sterility for hybrid breeding.

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