Theor Appl Genet (1987) 74:773–780

Molecular analysis of the mitochondrial genome of *Helianthus annuus* in relation to cytoplasmic male sterility and phylogeny

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Received May 11, 1987; Accepted May 22, 1987
Communicated by R. Hagemann

Summary. A circular supercoiled mitochondrial DNA plasmid P_1 (1.45 kb) is shown in both normal fertile plants of *Helianthus annuus,* and some cytoplasmic male sterile lines (CMS A and CMS P). In contrast, no plasmid is found in some other types of CMS C, I, B and K. A circular supercoiled DNA (P_2) of higher molecular weight (1.8 kb) is observed in CMS F. The mitochondrial plasmid P_1 was cloned, nick-translated and hybridized with native mitochondrial DNA from different lines of male fertile, CMS or wild *Helianthus.* No sequence homology has been detected between plasmid DNA P_1 and high molecular weight mitochondrial DNA in any line examined. A slight hybridization occurs between plasmids P_1 and P_2. Thus, there is no apparent relationship between mitochondrial plasmid DNA and CMS or *Helianthus* species. On the contrary, each *Helianthus* CMS and male fertile strain can be characterized by digestion fragment patterns (Sal I and Bgl I). Analysis of mitochondrial DNA from wild *Helianthus* strains indicated a relation between some CMS and the strain from which they were maternally derived, as for example CMS I and *H. annuus* ssp *lenticularis* and CMS F and *H. petiolaris fallax.* On the basis of restriction endonuclease patterns, a CMS phylogenic tree is proposed which illustrates a molecular polymorphism in the mitochondrial genome of *Helianthus.*

Key words: Mitochondrial DNA – Cytoplasmic male sterility – *Helianthus annuus* – Mitochondrial plasmid like DNA – Phylogeny

Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait which causes pollen abortion, is an incompability interaction between nucleus and cytoplasm. This characteristic is used in commercial hybrid seed production. Restorer genes are necessary in crops where the hybrid generated from a CMS parent is grown for its seed. The relations between male sterile-cytoplasms and their respective and specific nuclear fertility restoration genes (Rf) could be interpreted as complementary interaction resulting in either normal or abortive pollen.

The first reliable sunflower CMS was reported by Leclercq (1969) from an interspecific hybrid between *Helianthus petiolaris nutt* and the cultivated sunflower *H. annuus* L. In order to introduce cytoplasmic diversity into the crop, breeders have made attemps to find new sources of sunflower CMS. CMS has arisen in intraspecific crosses (*H. lenticularis* ssp *annuus* × *H. annuus* L.: KOUBAN CMS, Anashencko 1974; INDIANA CMS, Heiser 1982) as well as interspecific crosses (*H. petiolaris* × *H. annuus* L.: PETIOLARIS CMS, Whelan 1980; Fallax CMS, (Serrieys and Vincourt 1987; *H. Bolanderi* × *H. annuus* L.: Bolanderi CMS, Serrieys).

The pattern of restoration of male fertility in the presence of CMS has been until recently, the geneticist's primary method to distinguish cytoplasms, by test crosses with defined lines (Leclercq 1983, 1984). With the advent of molecular techniques which permit direct examination of cytoplasmic genomes, cytoplasms can now be differentiated further (Leroy et al. 1985). Two cytoplasms with indistinguishable reactions given restorer genes can be distinguished if their cytoplasmic genomes exhibit restriction site heterogeneity. Difficulty arises from the complexity and variability among mitochondrial genomes even of closely related species or varieties of the same species. The mitochondrial genome of different plant species with male-fertile and male-sterile cytoplasms differ in their restriction endonuclease patterns and in the number and type of

LMW mtDNA molecules that they bear: *Sorghum* (*Sorghum bicolor* L.) (Pring et al. 1982); *Vicia faba* L. (Boutry and Briquet 1982); *Brassica* (Vedel et al. 1982; Palmer et al. 1983); maize (*Zea mays* L.) (Kemble et al. 1983; Levings and Pring 1976; Pring and Smith 1985); sugarbeet (*Beta vulgaris* L.) (Powling and Ellis 1983; Mikami 1984); sunflower (*Helianthus annuus* L.) (Leroy 1985; Brown et al. 1985).

In this report, we characterize the mitochondrial DNA CMS in a series of different sunflower lines and in some isolates of wild *Helianthus* species in order to establish molecular analysis of sunflower CMS and in addition to provide an approach to the phylogeny of the *Helianthus* mitochondrial genus. A minicircle of 1.45 kb had been cloned and may be used as a probe.

Materials and methods

Plant material

Seeds of sunflower were kindly provided by F. Vear, P. Leclercq and J. P. Philippon (INRA, Station d'Amélioration des plantes, 63039 Clermont-Ferrand, France) for fertile *Helianthus annuus* (♂), french CMS (♀C), kouban CMS (♀K), CMS of unknown origin (♀A) and *H. argophyllus;* H. Serrieys (INRA, Station d'Amélioration des plantes, 34130 Mauguio, France) for Bolanderi CMS (♀B), Fallax CMS (♀F) and wild strains of *Helianthus annuus:* 114, 116, 120, 209, 210, 388, 421 and *Helianthus petiolaris* 199, 200; C. B. Heiser (Department of Biology, Indiana University, Bloomington, IN 47405, USA) for Indiana CMS (♀I) and wild *Helianthus annuus* 581, 8206. Plants were grown for at least 1 month in growth chambers at 23 °C under 10,000 lux daylight (16 h light/8 h dark) and 85% relative humidity.

Isolation of plant mitochondria

For this, 12 g of leaves from a single plant were cut, washed and homogenized for 2 × 3 sec at low speed in a two speed waring blender with 60 ml buffer V (0.6 M KCl, 0.05 M Tris-HCl pH 7.2, 0.02 M EDTA, 0.3% bovine serum albumine, 0.02 M 2-mercaptoethanol, 20 mM Diethyl carbanic acid; Sigma). The preparation was filtered through two layers of blutex (100 and 60 μm) prior to centrifugation for 5 min at 4,000 rpm in a Sorvall SS 34 rotor. The supernatant was then centrifuged for 10 min at 16,000 rpm in the same rotor to pellet the mitochondria. This pellet was resuspended with a small paint brush in 3 ml buffer R (0.3 M KCl, 0.05 M Tris HCl pH 7.2, 0.1% BSA, 0.02 M Magnesium acetate) and treated with 200 μg/ml bovine pancreatic DNAse (Sigma). After 20 min at 37 °C, the mitochondria were diluted with 10 ml of buffer NET (0.6 M NaCl, 0.05 M EDTA, 0.05 M Tris-HCl pH 8), and pelleted as described above.

Purification of mitochondrial DNA

Helianthus mitochondria were purified according to a modified method described by Wilson and Chourey (1984). Pellets of purified mitochondria were resuspended in 1.8 ml buffer NET, then 0.2 ml of 10% SDS in buffer NET was added and the mixture incubated for 20 min at 65 °C. After this, 0.7 ml 5 M potassium acetate were added and incubated for 15 min at 0 °C. The mixture was centrifuged for 20 min at 14,000 rpm (rotor SS 34) at 4 °C. The supernatant was collected and mixed with 1 ml 7.5 M ammonium acetate and 6 ml cold isopropanol. After 10 min incubation at room temperature, the mtDNA was precipitated for 30 min at 14,000 rpm in a 15 ml siliconed corex tube. The mtDNA pellet was finally resuspended in 20 μl of TE buffer (0.001 M EDTA, 0.01 M Tris-HCl pH 8.0).

Endonuclease digestion and agarose gel electrophoresis

Restriction endonucleases were obtained from Boehringer Mannheim and mtDNA samples (about 2 μg DNA) were digested with 50 units of enzyme for 2 h to 5 h at 37 °C with Sal I or Bgl I in appropriate buffer (Manniatis 1982). RNAse (DNAse free) was added to all restriction reaction digests. The samples were loaded in 1% agarose gels, electrophoresis was performed at 50 volts for 24 h for long size gels and 60 volts for 2 h for small size gels. Gels were stained with ethidium bromide and photographed on polaroid type 55 film under UV light. The reference size markers were purchased from BRL.

Cloning of 1.45 kb plasmid DNA and hybridizations

Minicircular DNA (1.45 kb) isolated from a male fertile strain (♂) of *Helianthus annuus* was cleaved by Eco RI mixed and ligated with the plasmid PUC 9 also digested by Eco RI and cloned in *E. coli* strain NM 522. Cloned recombinant plasmid DNA was extracted according to Holmes and Quigley (1981). The mitochondrial plasmid insert was then recovered by digestion with Eco RI, separation in agarose gel and electroelution of the insert DNA in Biotrap apparatus. The mitochondrial plasmid was then nick-translated using the BRL kit. Mitochondrial DNA in agarose gel was transferred to nylon Hybond N (Amersham) using the procedure of Southern (1975).

Prehybridization of membranes was performed at 63 °C for 2 h in a hybridization buffer consisting of 1 × Denhardt's solution in 3 SSC (0.45 M NaCl, 0.045 M sodium citrate pH 7.0) containing 50 μg/ml heat denatured herring sperm DNA and 0.1 ml SDS 20%. The denatured labelled DNA probes were injected then incubated for 30 h at 63 °C. Hybridized membranes were washed twice in 3 × SSC, 0.1% SDS for 45 min and twice in 0.2 SSC, 0.1% SDS for 45 min (all at 63 °C). After hybridization the filters were autoradiographed for 1 h to a few days at −70 °C.

Estimating sequence divergence, phylogenetic tree construction of mtDNA in some fertile and CMS H

Sequence divergence of mitochondrial DNA was estimated by the method of Nei and Li (1979). F values were then utilized to produce an evolutionary tree.

Results

Relation of minicircle to other mtDNA molecules

Sunflower mitochondria contain small, supercoiled DNA molecules in addition to the HMW mtDNA (Leroy et al. 1985). Two minicircles are distinguished by size. Their approximate sizes are 1.45 kbp (P_1) and 1.8 kbp (P_2). The mitochondria of male-fertile sunflower and CMS A and CMS P contain the P_1 plasmid; CMS F contains the P_2 plasmid. Four CMS (C, K, I, B) have no LMW minicircles (Fig. 1 A, Table 1).

These results are confirmed by hybridization experiments using cloned P_1 plasmid as a probe (Fig. 1 B).

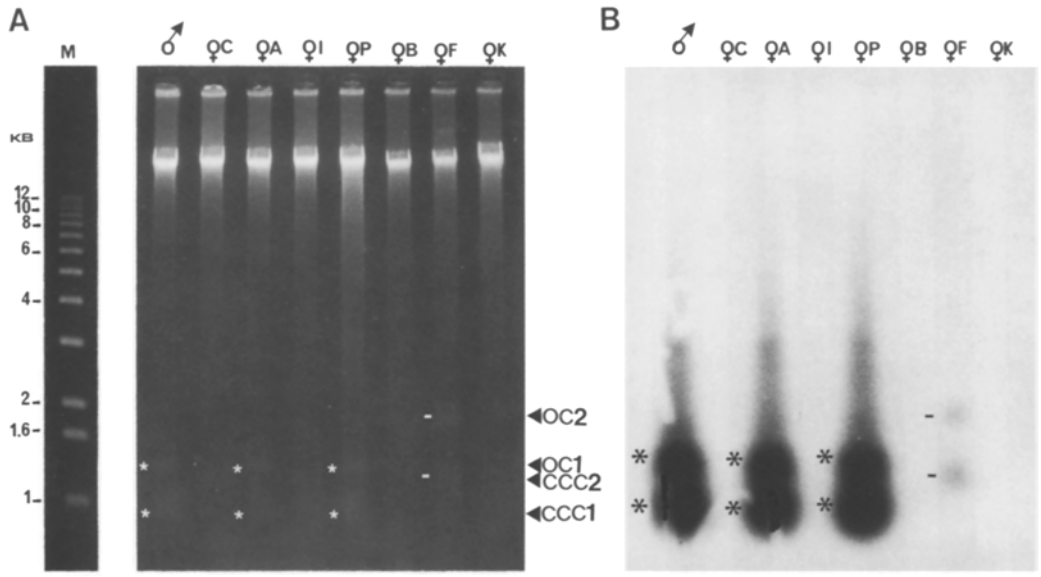

Fig. 1 A, B. Sunflower mitochondrial DNA analysis. **A** Agarose gel electrophoresis of native mitochondrial DNA from fertile, sterile sunflower (1% agarose slab gel and ethidium bromide staining). **B** Autoradiography of the hybrids of ^{32}P-labelled plasmid (1.45 kb) to mtDNA blotted onto nylon membrane. *Number left* indicates the fragment sizes in kb of standard molecular weights. OC_1 and OC_2 are open circular derivates respectively of CC_1 and CC_2 supercoiled plasmids P_1 (1.45 kb) and P_2 (1.8 kb). ♂: fertile sunflower; ♀ *C*: french CMS; ♀ *A*: unknown CMS; ♀ *I*: Indiana CMS; ♀ *B: H. bolanderi* CMS; ♀ P: *H. petiolaris* CMS; ♀ F: *H. petiolaris fallax* CMS; ♀ *K:* kouban CMS

Table 1. Localization of mitochondrial plasmids P_1 (1.45 kb) and P_2 (1.8 kb) in fertile and different CMS lines, as Fig. 1

	No. plasmid	Plasmid (1) 1.45 kb	Plasmid (2) 1.8 kb
♂		×	
♀C	×		
♀A		×	
♀I	×		
♀B	×		
♀P		×	
♀F			×
♀K	×		

We were not able to detect any hybridization with HMW mitochondrial DNA in our experiments, suggesting that no sequences homologous with the minicircles are present in the HMW mtDNA. Partial hybridization can be detected with plasmid P_2 (1.8 kbp) from CMS F, suggesting a slight sequence homology between the two plasmids. In all three cases of positive hybridization (fertile sunflower, CMS A and P) the cloned DNA hybridized to a series of molecules of increasing molecular weight. Comparison of their mobilities with those of the marker fragments suggests that these molecules are the dimers, tetramers, etc. of the original minicircle.

Comparison of the restriction endonuclease digestion patterns of mtDNA from normal and male-sterile cytoplasms of sunflower

A comparison of the restriction endonuclease digestion patterns of normal and CMS mtDNA showed that these cytoplasms possess their own characteristic pattern (Figs. 2 and 3). In Sal I digestion, normal and CMS mtDNA produced at least 35 to 38 discrete bands and with Bgl 1 29 to 32 bands with molecular weights ranging from 2 to 22 kbp. Some of the bands in the gel pattern are more intense than others and may represent either repeat sequences in the mitochondrial genome or overlapping fragments which are indistinguishable by mobility. Although most DNA fragments are common to all cytoplasms, each cytoplasm is characterized by fragments peculiar to that cytoplasm. The CMS C, A and K are closely related with normal line mtDNA, whereas CMS I, B, P and F are less related (Figs. 2 and 3).

Phenogram of different fertile and sterile cytoplasms using restriction fragments of mtDNA

We have examined the relationship between the mitochondrial DNA of fertile and 7 CMS strains of sunflower, using a graphic representation of shared fragments in Sal I and Bgl 1 digests. The calculations used to construct a phylogenetic tree rely on the assumption that no

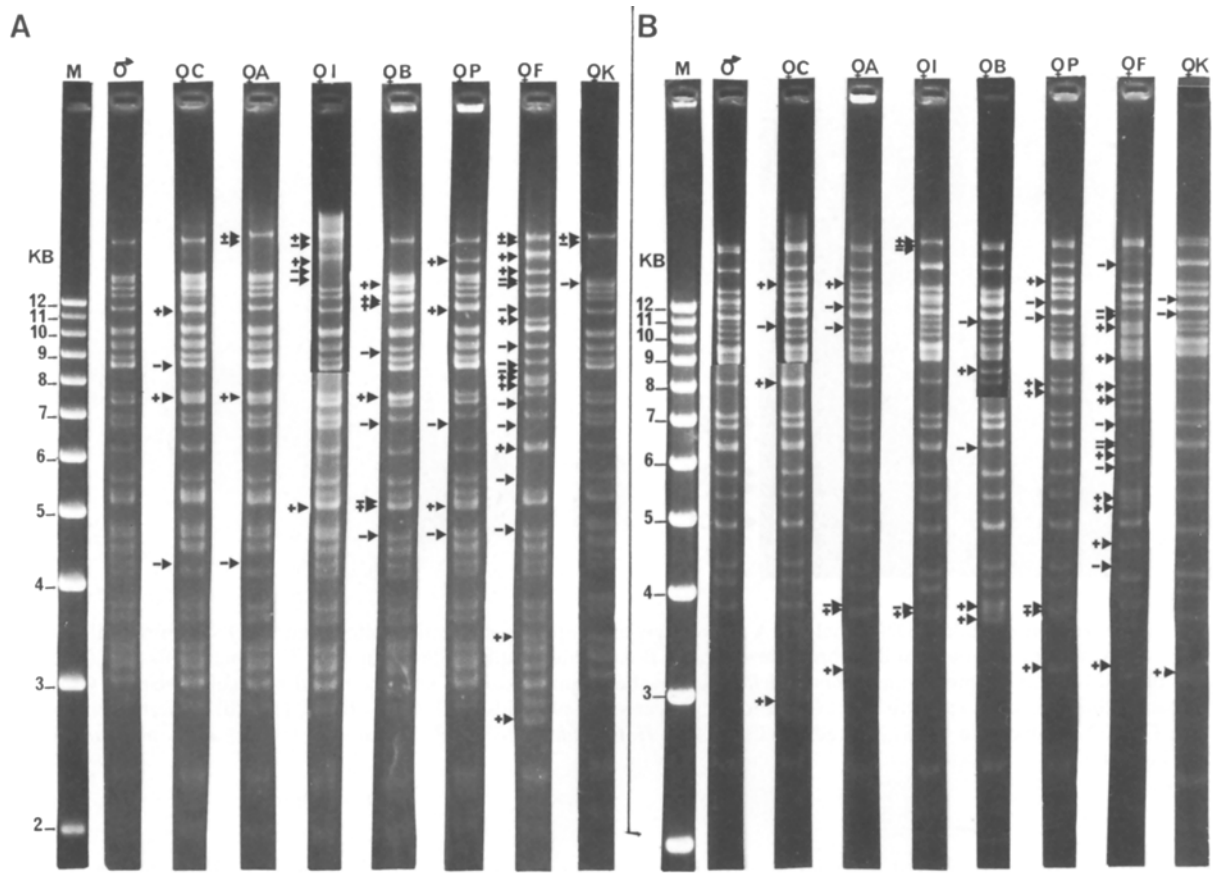

Fig. 2. Restriction analysis of various sunflower mtDNAs. Horizontal 1% agarose gel electrophoresis of restriction enzyme analysis of fertile and male-sterile mtDNAs. MtDNAs were digested by endonucleases Sal I (**A**) and Bgl I (**B**). *Lane M*: marker fragments as in Fig. 1.; ♂, ♀C, ♀A, ♀I, ♀B, ♀P, ♀F, ♀K are the same as in Fig. 1. *Arrows* are either more (+) or less (–) intense than fertile sunflower (♂)

rearrangements have occurred, a condition not yet seen in plant mtDNA. However, as a large fraction of the genome is conserved and a large data set was available, the comparison is still of interest and significance as an index of similarity (Borck and Walbot 1982). The F values summed over all enzymes were used as measures of similarity to generate the phenogram presented in Fig. 4. Two comments can be made. (1) The phylogenetic distribution of the mitochondrial plasmid observed only sporadically corresponds to a mitochondrial DNA based *Helianthus* phylogeny. (2) There is no correlation between the F values and maternal origin of CMS.

Molecular heterogenety in mtDNA of wild H. annuus and several Helianthus species

Five out of eight samples of wild strains of *H. annuus* collected in various parts of North America showed the presence of a 1.45 kb plasmid (Fig. 5). Thus we are not able to correlate the seed origin and the presence of the plasmid. The comparison of restriction patterns of *Helianthus* mtDNA shows several features, given below.

Intraspecific analysis of Helianthus annuus

(a) Three wild strains of *H. annuus* have a similar Sal I digestion pattern but can be characterized by the presence (isolate 114) or absence (strains 8206 and 121) of plasmid P_1 (Fig. 5).

(b) Three wild strains (120, 209, 581) have different digestion patterns to the reference fertile strain. We can observe that the electrophoretic pattern mtDNA Sal I of strain 581 (*H. lenticularis* ssp *annuus*) is similar to that of CMS Indiana and would suggest a same maternal parent between the two strains (Figs. 3 and 6).

Interspecific analysis

The analysis of *H. argophyllus* mtDNA shows a close similarity in electrophoretic pattern of restriction fragments to control fertile *H. annuus* (Fig. 6) which confirms the close relationship between the two species (Schilling and Heiser 1981).

The analysis of two strains of *H. petiolaris* (Fig. 6) indicate that some isolates of this specie (strain 199)

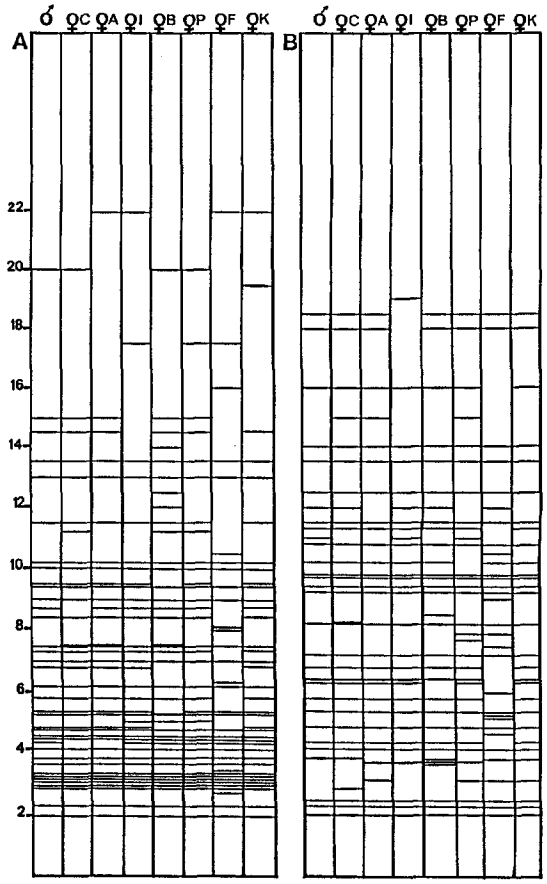

Fig. 3. Schematic diagrams of Sal I (**A**) and Bgl I (**B**) digests of fertile or sterile sunflower as in Fig. 2. Molecular weights (kb) are on the *left*

may be closely related to *H. annuus.* The strain *H. petiolaris* 200 shows an identical pattern to that of CMS F, using Sal I endonuclease, which demonstrates a conservation of mitochondrial DNA structure during crosses for obtaining this cytoplasmic male sterility.

Discussion

Considerable interest in mtDNA of higher plants has developed in recent years, especially in the complexity of mitochondrial genomes, cytoplasmic male sterility in various plant systems, molecular mapping of mitochondrial genomes and molecular polymorphism by restriction fragment profiles (Hanson and Conde 1985).

The procedure for isolation of sunflower mitochondria described here is much quicker and simpler than earlier methods. In addition, this method is not destructive. We can characterize the mitochondrial genome of a single plant without detroying it, thus remitting study of maternal lineage of the plant and allowing real genetic research. This procedure can be used to obtained restriction patterns from both wild and cultivated sunflower mtDNA.

A circular supercoiled 1.45 kb plasmid DNA, previously described in mitochondria of fertile *H. annuus,* was not detected in mitochondria of an isogenic CMS line (CMS C) containig cytoplasm from *H. petiolaris* in the presence of an *H. annuus* nuclear background (Leroy et al. 1985). Fertility restoration by nuclear genes had no effect on the absence of the plasmid (Brown et al. 1986). The same situation occurs in CMS K, I, B

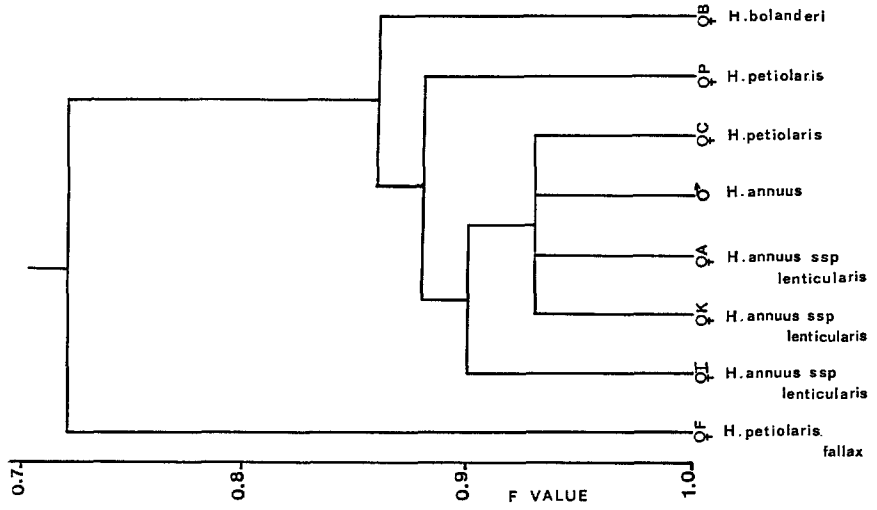

Fig. 4. Phylogenic diagrams of fertile and CMS sunflowers. The fraction of shared fragments was calculated as described by Nei and Li (1979) using the equation $F = 2n_{xy}/(n_x + n_y)$ in which F = the fraction of shared fragments, n_{xy} = the number of shared fragments, n_x the number of fragments analysed in one cytoplasmic type and n_y = the number of fragments analysed in the other cytoplasmic type. The origin of each cytoplasm is indicated, as is the possibility of mitochondrial plasmid P_1 or P_2

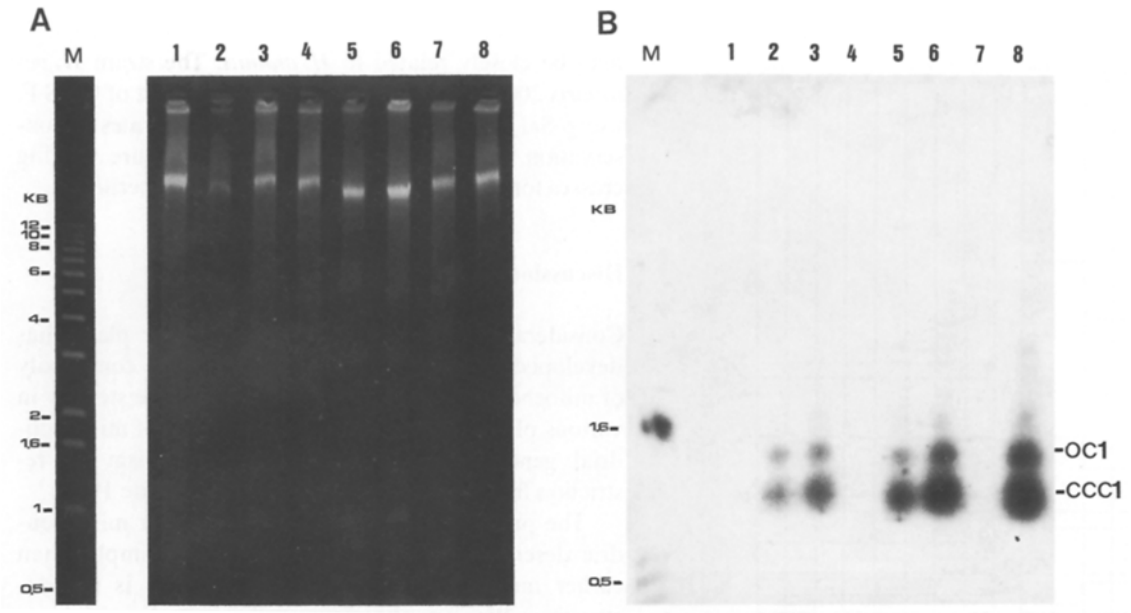

Fig. 5A, B. Southern hybridization analysis of mitochondrial DNA. **A** 1% agarose gel electrophoresis of native mitochondrial DNA from different wild *Helianthus annuus.* The electrophoregram of total mtDNA was stained with ethidium bromide. **B** Autoradiography of the hybridization of ^{32}P-labelled plasmid (1.4 kb) to mtDNA blotted onto nylon membrane. *Lane M* is marker fragments as in Fig. 1. Origins of *Helianthus annuus* used: *1*: 209 from Texas (USA); *2*: 120 from Canada; *3*: 210 from Texas (USA); *4*: 114 Canada; *5*: 8,206 from USA; *6*: 421 from California (USA); *7*: 388 from California (USA); *8*: 116 from Canada

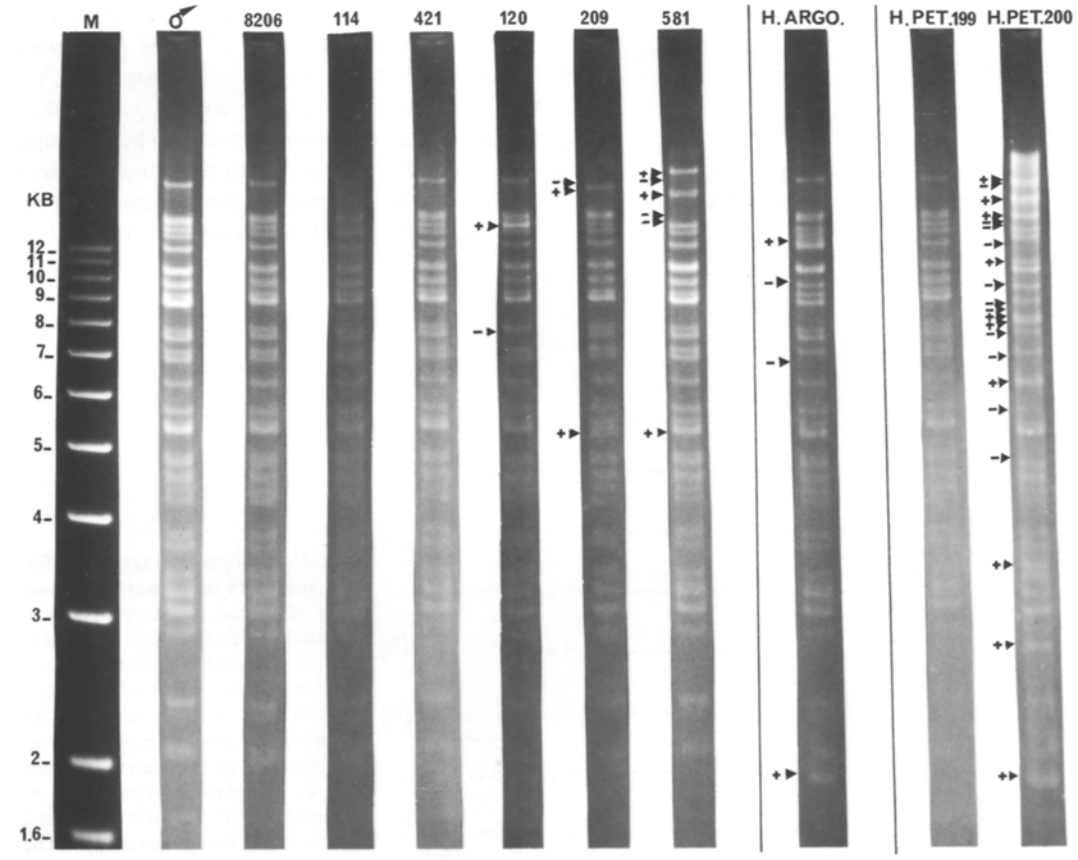

Fig. 6. Restriction enzyme analysis of different *Helianthus* mtDNA digested by endonuclease Sal I (1% agarose gel). *Lane M* is marker fragment as in Fig. 1. ♂: cultivated *H. annuus;* 8206, 421, 209, 581: wild *H. annuus* from USA; 114, 120: wild *H. annuus* from Canada; also shown are *H. argophyllus, H. petiolaris* 199 and *H. petiolaris fallax* 200. Bands with *arrow* are either more (+) or less (–) intense than cultivated sunflower

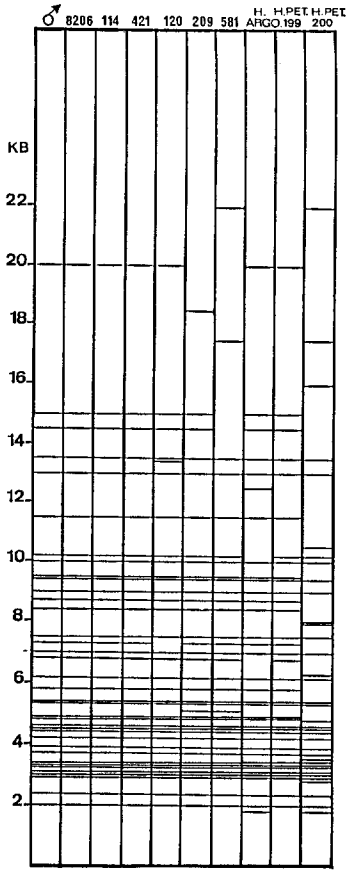

Fig. 7. Schematic diagrams of Sal I digests of different *Helianthus* as in Fig. 6. Molecular weight (kb) references are on the *left*

whose origins are, respectively, *H. lenticularis* ssp *annuus* for the former two and *H. bolanderi* for the latter. However, in two types of CMS: P and A with origins *H. petiolaris* and unknown respectively, a 1.45 kb plasmid DNA was detected. Thus, there appears to be no direct correlation between CMS and the presence of plasmid DNA

Further analyses with other *Helianthus* species (Figs. 1, 5) also demonstrate no correlation between mitochondrial plasmid and specific origin.

A related plasmid (P_2 : 1.8 kb) of greater size was found in fallax cytoplasm with an *H. annuus* nuclear background (CMS F). Hybridization experiments using a cloned 1.45 kb plasmid has shown sequence homology with the 1.45 kb plasmid isolated from various CMS or sunflower strains. A partial sequence homology occurs with the 1.8 kb plasmid and no sequence homology with the main mitochondrial DNA. A similar situation was described in *Beta* in which mitochondria with four discrete DNA molecules of 1.3, 1.4, 1.45 and 1.5 kb were detected (Powling 1982). The 1.45 and 1.5 kb circular molecules are related but the principal genome shared no homology with these minicircular DNAs (Powling and Ellis 1983). In *Vicia faba* mitochondria, three of the four plasmids analysed are closely related (Goblet et al. 1983). These minicircular mitochondrial DNAs, on the contrary, hybridized not only with their derivates, but also with some discrete classes of higher molecular weight DNA and with the major DNA mt (Negruk et al. 1985).

Small plasmid-like DNA molecules are found in the mitochondria genomes of many higher plants (Sederoff 1984). Frequently the number and types of such DNA differ between male fertile and male sterile cytoplasms but this correlation between CMS and plasmid presence is not observed in sunflower cytoplasms.

Restriction enzyme analysis of mtDNA of the male fertile and male sterile cytoplasms with Sal I and Bgl I has revealed fragment mobility differences and confirmed the data previously described (Leroy et al. 1985). The restriction patterns characterize specific sunflower CMS. It must be emphazised that the male sterile cytoplasms originate from different species of wild *Helianthus* (*H. annuus* ssp *lenticularis, H. petiolaris, H. bolanderi*). Male fertile cytoplasm is found in cultivated sunflowers which have undergone quite a long period of selection. The differences in mtDNA patterns could be due to these different origins and may not in fact have any causal relationship with the CMS trait.

Mitochondrial DNA of two types of CMS (CMS I und CMS F) show the same electrophoretic restriction patterns (Sal I) as those for the mtDNA from the *Helianthus* species from which the CMS cytoplasms originated (*H. annuus* ssp *lenticularis:* 581 and *H. petiolaris fallax*) respectively (Figs. 2 and 6). The fact that these wild strains, apparently containing CMS cytoplasms, show normal male fertile phenotypes, suggests that they must also contain nuclear restorer genes. This hypothesis was evoked by Leclercq (1971). Thus, the CMS phenotype does not appear to alter the physical map of mitochondria of a sunflower species and the evolution of the mitochondrial DNA structure appears to be independent of the nuclear environment. Moreover, the 1.45 kb mitochondrial plasmid is only observed in the mitochondria of male fertile cytoplasm (CMS-maintainer of ♀ C), indicating strict maternal inheritance of sunflower mtDNA.

The male fertile and male sterile mtDNAs of *faba* beans (Boutry and Briquet 1982) and Maize (Pring and Levings 1978; Borck and Walbot 1982) are thus considerably more divergent than those of sunflower. Most of this variation is unlikely to be related to the male sterile phenotype. Different studies have already suggested that classification of taxa based upon similarities and differences of organelle DNA correlate well with divisions arrived at by more traditional means (Weis-

singer et al. 1982). Moreover this idea cannot account for different *Helianthus* cytoplasm which have given the CMS types analysed. A phenogram (Fig. 4) summarizes the data of CMS isogenic strains of *H. annuus* with different CMS and shows the evolutive distance between some mitochondrial DNAs.

Acknowledgements. We are grateful to M. T. Gerles for her skillful assistance. This work is supported by a grant from "Ministère de la Recherche et de la Technologie". Biohazards associated with the experiments described in this publication have been examined previously by the French National Control Committee.

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