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Mitochondrial DNA diversity and male sterility in natural populations of *Daucus carota* ssp *carota*

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Abstract Mitochondrial variability was investigated in natural populations of wild carrot (*Daucus carota* ssp *carota*) in different regions: South of France, Greece, and various sites in the Mediterranean Basin and Asia. Total DNA was digested with two restriction endonucleases (*Eco*RV and *Hin*dIII) and probed with three mitochondrial DNA-specific genes (*cox*I, *atp*6, and *cox*II). Twenty-five different mitochondrial types were found in 80 analyzed individuals. Thirteen mitotypes were found among the 7 French populations studied. On average, 4.4 different mitotypes were observed per population, and these mitotypes were well-distributed among the populations. All of the mitochondrial types were specific to a single region. However, the proportion of shared restriction fragments between 2 mitotypes from different regions was not particularly lower than that which occurred among mitotypes from a single region. On the basis of the sexual phenotype [male-sterile (MS) or hermaphrodite] of the plants studied in situ and that of their progeny, 2 mitotypes were found to be highly associated with male sterility. Eighty percent of the plants bearing these mitotypes were MS in situ, and all of these plants produced more than 30% MS plants in their progeny. This association with male sterility was consistent in several populations, suggesting an association with a cytoplasmic male-sterility system. Moreover, these two mitotypes had very similar mitochondrial DNA restriction patterns and were well-differentiated from the other mitotypes observed in wild plants and also from those observed in the two CMS types already known in the cultivated carrot. This suggests that they correspond to a third cytoplasmic sterility.

Key words RFLP · Mitochondrial DNA diversity · Natural populations · Cytoplasmic male sterility · *Daucus carota* L.

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Introduction

Gynodioecy, the co-occurrence of male-sterile (MS) (i.e., hermaphrodites having lost their male function) and hermaphrodite plants in natural populations of a single species, is a widespread breeding system in the angiosperms (Delannay 1978; Kaul 1988). In many such species, expression of the sexual phenotype is the result of an interaction between nuclear and cytoplasmic genes (Couvet et al. 1990). Due to the efficiency of the MS system in hybrid seed production, the genetic basis of cytoplasmic male sterility (CMS) has been studied in many crop species (Hanson and Conde 1985). As a result, it is now known that major rearrangements in the mitochondrial DNA (mtDNA) are frequently characteristic of MS lines (Newton 1988; Hanson 1991), suggesting that the mitochondrial genome may play an important role in the determination of CMS.

At present little is known with respect to the amount of mtDNA diversity in natural populations of plants. Studies of interaspecific variation exist but are mostly confined to a limited number of cultivars or accessions rather than to natural populations (Palmer 1992). There are still fewer studies which have quantified mtDNA diversity within populations. Since mtDNA rearrangements are usually involved in the determination of male

sterility, gynodioecious species can be predicted to show mtDNA diversity, even at the population level. This has been confirmed in the few gynodioecious species that have been investigated in this respect (Van Damme 1986; Boutin et al. 1987; Belhassen et al. 1993; M. Tarayre unpublished data). Moreover, correlations between the sexual phenotype and the mitochondrial type of the plants have been found in these species (Van Damme 1986; Belhassen et al. 1993; Cuguen et al. 1994). Thus, population level surveys of the relationship between sexual phenotypes and mtDNA polymorphism should provide a useful means of determining the involvement of particular cytoplasmic types with CMS, and thus find new sources of CMS potentially useful for germ plasm diversification.

In the cultivated carrot (*Daucus carota* L. ssp *sativus*), two morphologically different CMS types are known: the 'Brown anther' type characterized by shrivelled, yellow-to-brown anthers (Banga et al. 1964) and the 'Petaloid' type, found in an American wild carrot population and which presents an additional set of green petals (Thompson 1961). These two male sterilities are functionally different and are controlled by different systems of nuclear-cytoplasmic interactions (Thompson 1961; Hansche and Gabelman 1963). It is also known that numerous mtDNA differences exist between the two morphological types and also between CMS types and fertile 'Normal' cytoplasmic types (Ichikawa et al. 1989; Pingitore et al. 1989; Sheike et al. 1992; Steinborn et al. 1992) and that there is relatively little diversity within each of the CMS mitotypes as well as within the 'Normal' mitotype (Pingitore et al. 1989). In wild carrot populations (*D. carota* ssp *carota*) located along the French Mediterranean coast, a survey of the frequency of MS phenotypes has shown that despite low frequencies within populations (ranging from 0 to 13%), male sterility occurs in 95% of the surveyed populations (J. Ronfort, unpublished data).

In this paper, we report on a study of mitochondrial variability in natural carrot populations at different geographical scales and examine the relationship between mitochondrial types and male sterility. MtDNA variation was characterized by Southern blot hybridization of total DNA to mtDNA sequences. By comparing the pattern of mtDNA diversity to phenotypic and geographic variation in natural populations, we discuss whether there is an association between the sexual form and the mtDNA types as is expected given the involvement of cytoplasmic types in the determinism of male sterility. Finally, we discuss whether such cytoplasmic types are similar to the two CMS that have previously been characterized in cultivated carrots.

Materials and methods

Plants studied

Wild plants (*D. carota* ssp *carota* L.) were sampled in 7 populations located in southern France, in a 100-km diameter area around Montpellier. The sex of each individual plant was recorded. Following in situ open pollination, seeds were collected on these plants, and 30 seedlings per family (i.e., per maternal plant) were grown under greenhouse conditions and used to estimate the sex ratios for each maternal plant (see Table 1).

The geographical range of this study was extended in two ways:

(1) seeds were collected from wild plants in natural populations in Crete (near Chania) and on the island of Paros (cyclad Islands), and from different sites across the Mediterranean Basin and in Asia (Table 1). A single random plant was chosen from each site. For these plants, the sexual phenotype of the original mother was not known, and we observed only hermaphrodites in their progenies.

(2) a sample of cultivated lines (*D. carota* ssp *sativus*) containing 3 individuals from the 'Brown anthers'-cultivated CMS type (Sa), 2 individuals from the 'Petaloid' CMS type (Sp), and 5 individuals carrying the so-called 'Normal' cytoplasmic type were obtained. A detailed list of this material is given in Table 1.

DNA isolations

Total DNA was isolated essentially as described by Dellaporta et al. (1983). MtDNA was isolated from 70 g of roots according to Boutry and Briquet (1982) with some modifications (Saumitou-Laprade et al. 1993). The isolation of chloroplasts and purification of chloroplastic DNA (cpDNA) were performed as described in Boutin et al. (1987) using 60 g of leaves.

DNA probes

Three mitochondrial probes were used: (1) The ATPase subunit 6 gene (*atp6*) of maize ATP synthetase (Dewey et al. 1985), (2) a 2.9-kb fragment of the *Oenothera* mitochondrial genome containing the cytochrome oxidase subunit 1 gene (*cox*I) and a 5′ adjacent sequence involved in the transcription regulation of the *cox*I gene (Hiesel et al. 1987), and (3) the so-called "*B9/91*" cloned fragment of carrot cytochrome oxidase subunit II (*cox*II) described in Lippok et al. (1992). *B9/91* is a 4.6-kb fragment flanked by two *Bam*HI restriction sites and contains two of the three reported *cox*II exons (Exons B and C), part of the common 5′ intron, the supplementary 3′ intron, and a 3′ flanking region. This homologous probe was kindly provided by A. Brennicke and B. Lippok. In order to identify eventual cross-hybridization signals between mt- and cpDNA, the purified cpDNA was also labelled and hybridized on the restricted total DNAs of all the individuals surveyed.

Restriction of total DNA

Total DNA was digested with either *Hin*dIII (8 U/μg) or *Eco*RV (4 U/μg) restriction endonuclease (for 3- and 2-h incubation period, respectively), with the addition of spermidine (2 m*M*) to the reaction buffer.

Southern blotting and hybridization

Two micrograms of digested DNA was fractionated by electrophoresis in 0.8% agars gels in a TAE buffer (40 m*M* Tris, 20 m*M* NaAcetate, 2 m*M* EDTA) and transferred to a nylon membrane (Biodyne A) using the vacuum-blot system of Pharmacia. Fixation was performed through UV cross-linkage (1.2 J/cm^2). Probes were labelled through random priming, without prior linearization, with digoxigenin d-UTP, and hybridizations were performed overnight at 68 °C according to the manufacturer's recommendation (DNA labelling and Detection Kit Non-radioactive, Boehringer Mannheim Germany). The resulting signals were detected through chemiluminescence (Allefs et al. 1990) using CSPD from Tropix. Each of these probes were hybridized against total DNA digests by each of the restriction endonucleases described above, leading to 6 endonuclease/probe combinations (E/P).

Table 1 Origin, location, male-sterile (MS) frequency in natural populations (sample size), and number of collected plants (MS + hermaphrodites) for the molecular analysis of *D. carota* L

	Population names and locations	MS frequency in populations (sample size)	Number of collected progenies (MS + hermaphrodites)
Southern France populations	Triadou	8 (52)	6 (3+3)
	Matelles	13 (106)	10 (7+3)
	Jacou	0 (105)	6 (0+6)
	Quissac	5 (124)	6 (3+3)
	St Guilhem	6 (102)	6 (4+2)
	Teyran	—	3 (3+0)
	Pic St Loup	7 (116)	13 (8+5)
Greek populations	ParosA		4
	ParosB		3
	ParosC		3
	Crete		4
Individual plants[a]	[(a)]Iran (wild)		1
	[(a)]India (wild)		1
	[(a)]Pakistan (wild)		1
	[(a)]Spain (wild)		1
	[(b)]Wir 2309 (wild)		1
Cultivars[b]	[(b)]Wir 1531 (cultivars)		1
	[(c)]Carole (Sa)[c]		1
	[(d)]Nandor (Sa)		1
	[(d)]Nanco (Sa)		1
	[(d)]Tino (Sp)		1
	[(c)]Danielle (Sp)		1
	[(c)]Duti 12–3 (Na)		2
	[(c)]8–12 (Np)		2
	[(d)]Nantaise d'Augagne (N)		1

[a] Individual plants were provided by: (a) the North Central Region P.I. Station, USDA, Iowa State University and (b) the N.I Vavilov, Scientific Research Institute of Plant Industry USSR (Leningrad)

[b] Cultivated plants (*Daucus carota* ssp *sativus*) with known cytoplasms were provided by: (c) the Research Institut I.N.R.A. and (d) breeders

[c] (Sa), (Sp), (Na), (Np) and (N) denote, respectively, CMS type 'Brown anthers' (CMSa), CMS type 'Petaloid' (CMSp), a cytoplasmic type associated with a maintainer line of CMSa, a maintainer line of CMSp and a 'Normal' cytoplasmic type of a male-fertile cultivar

For all E/P combinations, hybridization profiles observed on total DNA, cpDNA and mtDNA were compared in order to identify eventual cross-hybridization signals between mt- and cpDNA.

Data analyses

Similarity between the different mitochondrial patterns was assessed as the proportion of shared fragments between each pair of mitotypes after the formula $F_{XY} = (2 \times N_{XY})/(N_X + N_Y)$, where N_X and N_Y are the number of fragments identified in mitotypes X and Y, respectively, and N_{XY} the number of fragments common to the two types (Nei and Li 1979). F was calculated over all E/P combinations, and common fragments among hybridization patterns were assumed to be homologous. The resulting distance matrix $(1 - F_{XY})$ between mitotypes was used to generate a clustering tree according to the neighbor-joining algorithm using PHYLIP software (Phylogeny Inference Package) version 3.5p (Felsenstein 1993).

To quantify the revealed genetic diversity, the number of mitotypes per population (Ap) was computed (Nei 1978; Hamrick and Godt 1990). Differentiation among populations and regions was estimated through the parameter θ, according to Weir (1990, pp 145–152). This parameter θ is analogous to the conveniently used F-statistics of Wright (1951) with suitable corrections for haploid data. It measures the frequency differentiation of the mitotypes among populations. Its significance was assessed using a bootstrap procedure (Weir 1990, pp 140–143). In order to detect isolation by distance effects, the regression coefficient of $\log(M)/\log(d)$ was calculated, where M estimates the average migration rate among populations, $M = ((1/\theta - 1))/2$, and d is the distance between populations in kilometres (Slatkin 1993; Birky et al. 1989).

Results

Hybridization patterns and mtDNA diversity

Cross-hybridization of mitochondrial probes

To detect eventual sequence homology between total mt- and cpDNA, we pooled material from 'Brown anthers' cultivated plants to obtain purified mt- and cpDNA and total DNA. For each E/P combination, we compared the hybridization patterns obtained for these three DNA samples. For probes *atp*6 and *cox*II, the total DNA hybridization patterns strictly corresponded to those obtained for mtDNA, and there were no hybridization signals with cpDNA. Conversely, with the probe *cox*I, cpDNA signals were observed: among the five signals obtained on total DNA/*Eco*RV restriction fragments, only three were specifically provided by the mtDNA and two appeared on cpDNA. As expected, these two chloroplast bands (22 kb) were observed on the total DNA hybridization patterns of all the sampled individuals (for *Eco*RV/*cox*I), except in one case where only the 16.4-kb fragment existed. This exception indicates cpDNA polymorphism. With *Hin*dIII, of the

three bands obtained on total DNA, one (4.7 kb) corresponds to cpDNA and the two remaining ones to mtDNA. No polymorphism was detected in that case. These chloroplast signals are shown on Fig. 1. In order to restrict our analyses to specific mtDNA signals, the chloroplast bands were ignored in *cox*I hybridization patterns. In subsequent analyses, *cox*I variants correspond to these corrected patterns.

MtDNA polymorphism

Of the six E/P combinations analyzed only one, *Eco*RV/*cox*II, was monomorphic. The number of variants observed with the five remaining E/P combinations ranged from 6 to 13 (Table 2). A sample of the observed hybridization patterns are shown in Fig. 1. The hybridization patterns obtained with *Hin*dIII/*atp6* on the cultivated male-sterile ('Brown anther' and 'Petaloid' types) and fertile (Normal) cytoplasms enabled us to make comparisons with available data from Scheike et al. (1992): the same hybridization patterns were found for the three former cytoplasms, while wild plants revealed six new profiles (on Fig. 1b, the first hybridization pattern for *Hin*dIII/*atp6* corresponds to the 'Brown anther' and to the Normal cytoplasmic types; the second one corresponds to the 'Petaloid' cytoplasmic type). According to the description of the *cox*II gene in carrots (Lippok et al. 1993), the cloned fragment *B9/91* was expected to hybridize to four restriction fragments. All the restriction fragments we observed were of higher molecular weight (from 5.5 to 13 kb), showing that the individuals studied were all different in this genomic region from the accession used to obtain this probe.

By combining the five polymorphic E/P combinations, we could define 25 different mitotypes among the 80 plants surveyed (described in Table 2). Four E/P combinations were sufficient to distinguish these 25 mitotypes, with *Hin*dIII/*cox*I revealing no additional polymorphism. Among these 25 mitochondrial types, 13 (designated as W_1-W_{13}) were found in individuals sampled from populations located in the south of France (50 individuals) and 9 in Greek populations (G_1–G_6) and in various sites around the Mediterranean and Asia (F_1–F_3). S_a/S_p, and N_1 which correspond to the mitotypes of cultivated plants, were never found within the wild plants sampled. The same mitotype (designated Sa) was observed for the 2 'Brown Anther' individuals (from cvs 'Nandor', 'Carole', and 'Nanco', see Table 1). The two 'Petaloid' individuals (from cvs 'Danielle' and 'Tino') also shared the same mitotype (Sp). Two different mitotypes were found within the 'Normal' cytoplasmic type we studied: one of them (N_1) was associated to the maintainer lines of the 2 CMS lines (plants 8.12 (Np) and 'Duti 12-3' (Na), maintainer lines of 'Petaloid' and 'Brown anthers' lines, respectively); the second type (N_2)

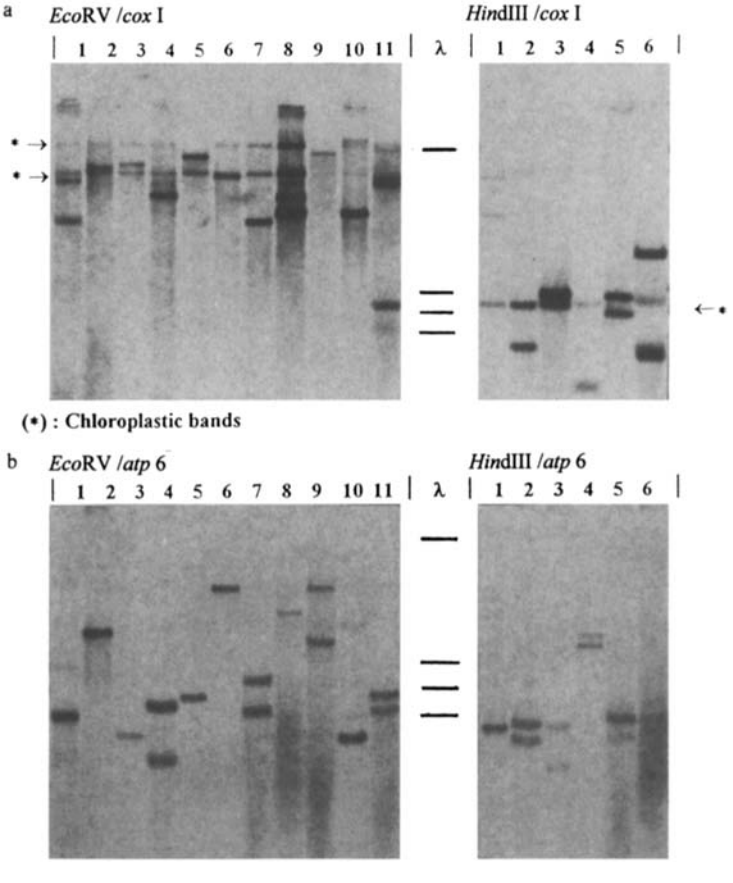


Fig. 1a,b Autoradiograms showing hybridization of the **a** *cox*I and **b** *atp6* probes to Southern blots of *Eco*RV and *Hin*dIII restriction fragments (to the *left* and *right* of the molecular weight marker, respectively) on *D. carota*. The designation above the tracks refers to the E/P combination variants as described in Table 3. Molecular weight marker: *Eco*RI-*Hin*dIII fragments of lambda DNA (fragment sizes from *top* to *bottom*: 21, 5, 4.3 and 3.5 kbp). *Arrows* indicate cp fragments on *cox*I hybridization patterns

Table 2 Description of the variants observed for each enzyme/probe (E/P) combination (lines) and of the 25 mitotypes defined by combining all the E/P combinations (columns) in *D. carota* L

		French populations													Greek populations						Foreign individuals			Cultivars			
Hybridization patterns (fragment sizes)	Mitotypes designations[a]	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	G1	G2	G3	G4	G5	G6	F1	F2	F3	Sa	Sp	N_1	N_2[c]
*Eco*RV/*cox*II																											
7.8	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Hin*dIII/*cox*II																											
10.5/5.5	1	+	+	+	+	+	+	+	+						+	+	+	+			+					+	+
6.5/5.5	2									+																	
10.0/5.5	3										+	+							+			+		+			
11.6/8.5	4												+	+									+				
5.5	5																			+							
13.0/11.3/9.2	6																								+		
*Eco*RV/*cox*I																											
13.9/12.5	1																						+				
16.8	2																+										
17.5	3																		+	+							
12.5	4							+	+																		+
18.4	5				+											+										+	
14.9	6										+	+						+				+					
9.9	7					+							+	+													
11.8/10.5	8								+	+																	
18.9	9	+	+	+											+												
10.5	10																								+		
20.3/14.9/13.9/4.2	11																							+			
18.9/18.4	12																				+						
*Hin*dIII/*cox*I																											
16.3/10.6	1																			+							
3.3	2						+	+					+	+									+				+
4.9	3								+	+																	
2.7	4	+	+	+	+	+					+	+			+	+	+		+		+	+				+	
2.9/7.4	5																							+			
4.4/5.1	6																								+		
2.9	7																	+									

Table 2 (Continued)

Hybridization patterns (fragment sizes)	Mitotypes designations[a]	French populations													Greek populations						Foreign individuals			Cultivars			
		W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	G1	G2	G3	G4	G5	G6	F1	F2	F3	Sa	Sp	N_1	N_2[c]
*Eco*RV/*cox*II																											
7.8	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Eco*RV/*atp*6																											
7.9	1										+																
3.6	2	+					+	+											+					+		+	+
7.5	3								+	+															+		
3.6/1.8	4				+																						
4.2	5											+								+		+					
12.0	6		+	+																							
4.8/3.6	7															+											
9.7	8																+										
17.2/8.5	9					+									+												
2.0	10												+										+				
4.2/3.6	11																				+						
7	12																	+									
3.0/2.7	13													+													
*Hin*dIII/*atp*6																											
3.2	1				+	+	+				+		+	+				+	+				+	+		+	+
3.2/2.7	2								+	+										+		+			+		
3.2/2.3	3											+															
5.9	4			+																							
3.2/2.6	5															+					+						
3.2/2.9	6																+										
6.5	7	+	+												+												
3.9/3.2	8							+																			

[a] W1–W13 refer to mitotypes found in the French natural populations, G1–G6 to those found in Greek populations (G2, G5 and G6 correspond to plants from the Paros Island, the others to plants from Crete), and F1–F3 to the various accessions provided by different research Institutes (F1 was found on the individual plant from Iran, F2 on plants from India, Pakistan, and USSR, and F3 on plants from Spain). Sa, Sp, N_1 and N_2 correspond to the cultivated cytoplasms ('Brown anthers', 'Petaloid' types, and two Normal fertile cytoplasms, respectively)

[b] For each E_p combination, the variant designations refer to hybridization pattern designations in Fig. 1

[c] Same mitotypes

was found either in cv 'Nantaise d'Aubagne' or within the French wild plants, and corresponds to the mitotype W_6 (see Table 2).

Differences between mitotypes

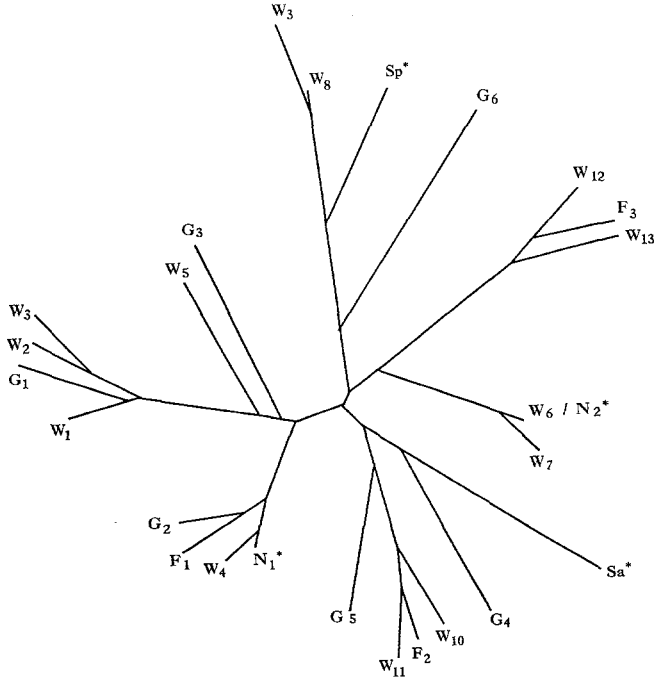
Differentiation was high between mitotypes: the mean distance between them based on the proportion of shared fragments ($1 - F_{XY}$) was 0.586 (SE = 0.012). Similarities can be visualized on the Neighbor-joining unrooted tree (Fig. 2). The two CMS cytoplasms 'Brown Anther' and 'Petaloid' (Sa and Sp), and the 'Normal' cytoplasm (N1 and N2) are located on three distinct branches ($1-F_{Sa/Sp}=0.789$; $1-F_{Sa/N}=0.625$; $1-F_{Sp/N}=0.6$; $1-F_{N1/N2}=0.333$). A fourth branch clearly sets apart 3 other mitotypes: W_{12}, W_{13} (from France), and F_3 (from Spain): $1-F_{Sa/W12}=0.76$, $1-F_{Sp/W12}=0.75$, and $1-F_{N/W12}=0.692$ and 0.538 for N1 and N2, respectively. In particular, these 3 mitotypes did not share any fragments, with an other group of mitotypes found on French wild plants (the group composed on W1, W2, and W3). Less marked differentiation among mitotypes was also observed, and several similar mitotypes were grouped at the end of the branches because they were different for only one or two E/P combinations.

Geographical distribution of the diversity

All of the mitotypes defined were specific to a single geographic region, with no single mitotype being found in two different regions (the three regions considered are France, Greece, and Crete). Nevertheless, no phylogeographic differences existed between these specific mitotypes (as can be seen on Fig. 2): distances ($1 - F$) between mitotypes of different regions ($1 - F = 0.604$, on average) are not higher than distances between mitotypes of the same region (Mean $1 - F = 0.605$ and 0.524 within the French and the Greek regions, respectively). On the basis of mitotype frequencies, the differentiation between regions was not significant ($\theta = 0.12$, $P > 0.10$).

The polymorphism observed in France was largely due to intra-population diversity. On average 4.4 mitotypes (SE = ± 0.72) were found per population for an average of 7 individuals studied per population (SE = 1.25). Most of the mitotypes were found in several populations. In particular, mitotype W12 was revealed in 5 of the 7 French populations (Triadou, Matelles, Jacou, Pic St Loup, Teyran). Differentiation in mitotype frequencies among the French populations was not significant: $\theta = 0.078$ ($P > 0.10$), as the regression coefficient of $\log(M)$ on $\log(d)$: $\beta = -0.45$ ($P > 0.30$).

Fig. 2 Neighbor-joining unrooted tree based on the similarity between *D. carota* mtDNA types ($1 - F_{XY}$ distance matrix) constructed with the PHYLIP software [Phylogenetic Inference Package, version 3.5 (Felsenstein 1993)]. Mitotype designations are those defined in Table 2)



Correlation between mitotypes and sexual phenotypes

For the 'Petaloid' CMS, a particular hybridization pattern was found for all of the polymorphic E/P combinations, compared to N_1, N_2, and Sa. For the latter three, similar variants were found with the probe *atp6* (as previously shown in Scheike et al. 1992), but distinct hybridization patterns were revealed for the E/P combinations.

The MS plants collected from natural populations in southern France were morphologically close to the cultivated 'Brown anther' CMS, i.e., shrivelled, yellow-to-brown, non-functional anthers were either clearly visible or hardly distinguishable. In contrast, no wild plant of the 'Petaloid' form has been found.

There was no strict relationship between sexual phenotype (MS versus hermaphrodite) and mitotype, for example, seven out of the ten patterns found on more than 1 plant were associated with both sexes. Nevertheless, the mitotype W12 was strongly associated with the MS phenotype: of the 9 individuals having this mitotype, only 1 was an hermaphrodite. We examined the proportion of MS plants in the progeny of 30 of the studied plants (25 MS and 5 hermaphrodites) after in situ pollination: it ranged between 0 and 62%. The relationship between these sex ratios and the mitotypes of the corresponding maternal plants is given in Table 3. Of the 11 mitotypes found on these plants, 9 appeared to be associated with either non-segregating plants or with giving few MS offsprings. All of the progenies containing more than 30% MS plants were, however, found on plants associated with mitotypes W_{12} or W_{13} (Table 3). Even the single hermaphrodite plant carrying W13 gave 55% MS plants in its progeny.

Table 3 Percentage of MS plants in open-pollinated progenies of maternal plants collected from natural populations of *D. carota* in Southern France according to their mitotypes. Each value corresponds to a single progeny of 28–37 plants

Mitotype of the mother plants	Sex of the mother plants	
	Hermaphrodite	Male sterile
W1	0^{a}	0^{e}-28.6^{a}
W2	0^{f}	0^{f}-5.7^{c}-18.2^{d}-18.7^{f}
W3		25.0^{e}
W4		0^{f}
W5		6.1^{b}-26.7^{b}-27.3^{b}
W6	0^{d}	12.9^{f}
W7		0^{d}
W10	9.4^{d}	18.9^{d}
W11	0^{f}	0^{b}
W12		61.8^{e}-41.9^{a}-40.0^{b}-40.0^{a} 36.1^{f}-33.3^{f}-33.3^{b}-32.2^{e}
W13	55.2^{f}	56.7^{f}

Maternal plants originated from populations: [a] Triadou, [b] Matelles, [c] Quissac, [d] St Guihelm, [e] Teyran, [f] Pic St Loup (see Table 1)

Discussion

Mitochondrial diversity and geographical variation in wild carrots

Although previous studies have shown that high differentiation of the mitochondrial genome exists between species and subspecies in the genus *Daucus* (Debonte et al. 1984; Ichikawa et al. 1989; Pingitore et al. 1989) and between CMS and fertile lines in the cultivated form *Daucus carota* L. ssp *sativus* (Steinborn et al. 1992; Scheike et al. 1992), the amount of variation observed in our study is particularly high given the low taxonomic level considered (i.e., within a subspecies and at the population level). Using only three mitochondrial genes as probes and two restriction endonucleases, we observed 25 different mitotypes among the 80 individuals analyzed.Thirteen mitotypes were found among the 50 individuals collected from French natural populations. Any available data concerning intraspecific variation in plant mtDNA mainly comes from studies of cultivated plants. As most of these report little variation, the idea has originated that between-species mitochondrial variation largely outweighs that within species (reviewed in Sederoff 1987). The study presented here shows that the mitochondrial genome may show a high level of intraspecific polymorphism, as has been reported in other wild species (Breiman et al. 1991; Khairallah et al. 1992; Luo et al. submitted; Saumitou-Laprade et al. 1993).

Because CMS implies mitotype variability, gynodioecious species can be predicted to show greater levels of mtDNA polymorphism than non-gynodioecious species. MtDNA variability has been observed in all the gynodioecious species investigated in this respect (Rouwendal et al. 1987; Boutin et al. 1987; Belhassen et al. 1991) and has been found to be particularly high in *Thymus vulgaris* (Belhassen et al. 1993; M. Tarayre unpublished data), a species that shows very high frequencies of male sterility (on average 60% but up to 95%) in natural populations. This suggests that the level of mtDNA variability could be related to the frequency of cytoplasmic male sterility a species displays. The results presented here do not confirm this predicted relationship. So, even in non-gynodioecious species or in species showing low levels of male sterility (frequencies of male sterility below 15% in *D. carota* L.), high levels of mitochondrial diversity can be found.

The majority of the variation we observed in *D. carota* was a consequence of intra-population diversity. Most of the mitotypes were found in several populations, and little among-population variation in mitotype frequencies was detected (as indicated by the low θ value). Studies concerning variation in the mitochondrial genome in samples of natural populations are scarce. In *Pinus* species, low mtDNA diversity (surveyed through four E/P combinations) exists within populations, at most 2 mitotypes were revealed per population (Strauss et al. 1993; Dong and Wagner 1993). In the gynodioecious species *Beta vulgaris* ssp *maritima*, 2–4 different mitotypes have been found within populations (Saumitou-Laprade et al. 1993). Compared to these species, the amount of diversity at the population level is high in wild *D. carota*. Compared to the level of population differentiation observed in French populations of carrots, higher Fst values have been found in *Pinus* species (ranging from 0.66 to 0.96, Trauss et al. 1993; Dong and Wagner 1993) and in *Beta vulgaris* ssp *maritima* (0.39, Cuguen et al. 1994). However, comparisons between studies are difficult because the geographic distribution of the sampled populations are highly variable between studies. For example, the sampled populations in the above two species were isolated by much larger distances (more than 100 km) than the populations considered here (all of the French populations were less than 50 km apart). For the cytoplasmic genome, population structure is related to the relative rate of seed migration and mutation, with low Fst values to be expected when seed migration is high (Cockerham and Weir 1993). Wild carrots are commonly found in recent fallows and are rapidly replaced by plants of a later succession stage, these frequent colonization and extinction events will increase gene flow. Furthermore, the capacity for long-distance dispersal is well-known in this species (Lacey 1980; Lacey 1982). As a result, natural populations of this species should show low levels of population differentiation.

On a wider scale (i.e., regional level), strong geographical specificity was found for all the mitotypes. However, no phylogeographic difference (Fig. 2) appeared because mitotypes of different regions often show similar restriction fragments. The occurrence of recurrent mutation events could explain this result and raises the question of the occurrence of homoplasic events in the mitochondrial genome (see also Strauss et al. 1993; Dong and Wagner 1993).

MtDNA polymorphism and sexual phenotypes

Due to the complex molecular structure of plant mtDNA and its poorly known mode of evolution, similarity estimations based on the proportion of shared fragments is a poor tool by which to infer phylogenetic relationships between mitotypes (Nei and Li 1979; Khairallah et al. 1991). The unrooted tree based on this measure does, however, provide indications concerning the types of variations observed. First, substantial differences involving several of the analyzed coding regions exist between the revealed mitotypes (as indicated by the long length of certain branches on the tree, Fig. 2). Such a high differentiation was revealed between Sa, Sp, and N and, in accordance with previous studies, suggests that the two specific CMS types are related to large mtDNA structural changes and that different functions may be involved in the pollen maturation defect (Scheike et al. 1992; Pingitore et al. 1989). Second, the occurrence of very similar mitotypes grouped at the end of the branches suggests that slight variations regularly occur within mitochondrial types. Such slight variations have also been reported within the two cultivated CMS types (Pingitore et al. 1989). This author found that based on variation in mtDNA restriction patterns (*Hin*dIII), the similaity between 2 CMS lines of the same morph was 91% for 'Petaloid' lines and 98% for 'Brown Anther' lines. Among the wild plants surveyed in the present study, high differentiation between groups of mitotypes also occurs. In particular, several rearrangements seem to be involved in the molecular differentiation of mitotypes W12 and W13.

On the basis of the proportion of MS plants observed in progenies following in situ pollination, two groups of mitotypes could be classified. Eleven mitotypes were associated with mother plants giving low levels of male sterility in their progenies. These mother plants included both hermaphrodites and MS individuals. In contrast, 2 mitotypes (W12 and W13) were most commonly found in male-sterile plants producing large numbers of male steriles in their progeny. On one hand, this association was a consistent feature of the 5 populations in which mitotype W12 occurred, on the other hand, the molecular patterns of mitotypes W12 and W13 were very similar to each other but very different to those of the other mitotypes found in the same natural populations (Fig. 2). These two results provide strong support for the idea that W12 and W13 are associated with cytoplasmically determined male sterility. At present, this assumption is strongly supported by unpublished results from crossing experiments in which 100% MS progenies have been obtained in crosses between MS plants carrying these mitotypes and cultivated maintainer lines and, also, in the first back-cross generation (J. Ronfort, unpublished data). Since mitotypes W12 and W13 have very similar hybridization patterns and co-occur in the same populations, they may correspond to a single CMS type. Although their hybridization patterns are clearly different from those of the two cultivated CMS types, it remains to be seen if they represent a third CMS in carrot or if they are functinally identical to either of the 'Brown anther' or 'Petaloid' CMS's. On the basis of the morphological characteristics of wild MS plants, they are likely to be different from the 'petaloid' CMS. The necessary controlled crosses are now in progress.

It remains to determine which kind of determinism is associated with the male-sterile phenotypes observed in the other mitotypes since CMS with corresponding restorer genes present at a high frequency in the populations, nuclear determination, or environmental factors could all be involved (see Kaul 1988). However, the use of molecular techniques for the screening of natural populations has proven to be an appropriate means to detect potential CMS in natural carrot populations that may be of use in plant breeding programs.

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