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The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp. *maritima* (L.): the usefulness of both genomes for population genetic studies

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Abstract

The structure and evolution of the plant mitochondrial genome may allow recurrent appearance of the same mitochondrial variants in different populations. Whether the same mitochondrial variant is distributed by migration or appears recurrently by mutation (creating homoplasy) in different populations is an important question with regard to the use of these markers for population genetic analyses. The genetic association observed between chloroplasts and mitochondria (i.e. two maternally inherited cytoplasmic genomes) may indicate whether or not homoplasy occurs in the mitochondrial genome. Four-hundred and fourteen individuals sampled in wild populations of beets from France and Spain were screened for their mitochondrial and chloroplast polymorphisms. Mitochondrial DNA (mtDNA) polymorphism was investigated with restriction fragment length polymorphism (RFLP) and chloroplast DNA (cpDNA) polymorphism was investigated with polymerase chain reaction PCR–RFLP, using universal primers for the amplification. Twenty and 13 variants for mtDNA and cpDNA were observed, respectively. Most exhibited a widespread geographical distribution. As a very strong linkage disequilibrium was estimated between mtDNA and cpDNA haplotypes, a high rate of recurrent mutation was excluded for the mitochondrial genome of beets. Identical mitochondrial variants found in populations of different regions probably occurred as a result of migration. We concluded from this study that mtDNA is a tool as valuable as cpDNA when a maternal marker is needed for population genetics analyses in beet on a large regional scale.

Keywords: cpDNA, mtDNA, organellar genome association, PCR–RFLP, wild beet

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Introduction

Organellar genomes — chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) — have been increasingly used in the past few years as markers to assess maternal or paternal gene flow because of their uniparental mode of inheritance (McCauley 1995). Nevertheless, the conjoint use of both markers is uncommon in the literature (but see Dumolin-Lapegue *et al*. 1998) although such an approach is of marked interest: (i) to detect paternal leakage of genomes assumed to be maternally inherited; (ii) to give insights into the usefulness of cpDNA vs. mtDNA for population genetics studies; and (iii) to evaluate the role played by recurrent mutations in organellar genomes. A particular mitochondrial haplotype found in several geographically distinct areas may result from either migration or recurrent mutational events. In the latter case (recurrent mutation), the geographical pattern is a result of homoplasic events (i.e. identity in state but not by descent). What is known so far of the plant mitochondrial genome structure strongly suggests the occurrence of such events among plant species.

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Homoplasy has been found, for example, in *Pinus* and *Hevea* (Strauss *et al*. 1993; Luo *et al*. 1995). Plant mitochondrial genomes differ markedly in size and structure from their animal counterparts. They vary in size from 200 to 2500 kb, with a complex multicircular structure (Fauron *et al*. 1995; Backert *et al*. 1997). Conversely to plant nuclear and chloroplast genomes, the plant mitochondrial genome displays a low rate of nucleotide substitution (Wolfe *et al*. 1987) and seems to evolve mainly in structure by intragenomic recombination through small repeated sequences dispersed within the genome (Lonsdale *et al*. 1988; Palmer 1992). However, because the number of small repeated sequences through which recombination events occur is rather low (five for *Beta*, see Lonsdale *et al*. 1988), the number of possible conformations of the mitochondrial genome will be limited. The same mtDNA genotypes could thus arise independently in distant populations. According to theoretical models (Atlan & Couvet 1993), several stable states, corresponding to different equilibria, could be reached, depending on the replication rates of the different subgenomic molecules created by recombination. However, a slight modification in the replication rate of one of the subgenomic molecules could shift the entire population of molecules to another equilibrium state, which may already be present in other plants. Because of such frequent structural mutations, mtDNA polymorphisms are frequently suspected of homoplasy, which has led to doubt regarding their usefulness for population biology studies (Parker *et al*. 1998). Conversely, the chloroplast genome is well characterized and its structure is very stable (Clegg *et al*. 1994). It has been widely used as a phylogenetic marker (Olmstead & Palmer 1994) but also, more recently, for within-species population genetics studies (see for example Taberlet *et al*. 1991; Ennos 1994; McCauley 1995; Dumolin-Lapegue *et al*. 1997a; Forcioli *et al*. 1998).

An important question is whether mtDNA could be as useful as cpDNA for population biology studies. First, it should be known whether a mtDNA genotype found in several populations is the result of migration among these populations, or has appeared independently in each population through independent mutations. In most higher plants, chloroplasts and mitochondria are both maternally inherited (Milligan 1992; Reboud & Zeyl 1994). As a result, if a given mtDNA type appears independently in different populations through recurrent mutations, it would probably be associated with different chloroplast genotypes. Conversely, the occurrence of the same combination (i.e. a given cpDNA haplotype together with a given mtDNA haplotype) in different populations is a strong indication for a low mutational process in both cpDNA and mtDNA genomes. As a consequence, the linkage disequilibrium between the two organellar genomes should be near its maximum value. This does not apply to gymnosperms, as they mostly show maternal transmission of mitochondria but paternal transmission of chloroplasts (e.g. Dong & Wagner 1994). In angiosperms, studies concerning the association between the two organellar genomes are very scarce. When both organellar genomes have been investigated, one of the two genomes often exhibited a limited polymorphism (an example is shown in Laurent *et al*. 1993), if not monomorphism (Wolf *et al*. 1997). Moreover, as pointed out by Dumolin-Lapegue *et al*. (1998), even when information on the two organellar genomes has been obtained in a species, 'the association between the two lineages has been not explicitly studied'.

B. vulgaris ssp. *maritima* is a biological model that is well suited for testing the usefulness of cpDNA and mtDNA as population genetics markers, and for investigating the association between both genomes. On the one hand, the use of mtDNA polymorphism is very common in beets, and large data sets are already available (Komarnitsky *et al*. 1990; Boudry *et al*. 1993; Cuguen *et al*. 1994; Senda *et al*. 1995). In addition, the occurrence of cytoplasmic male sterility involving the mtDNA genome has led to an increase in the use of mtDNA markers in this species, as well as in other gynodioecious species (e.g. Saumitou-Laprade *et al*. 1993; Ronfort *et al*. 1995). On the other hand, polymorphic cpDNA markers are also available in *B. vulgaris*. In a previous study, cpDNA was investigated by using restriction fragment length polymorphism (RFLP) Southern variants (Forcioli *et al*. 1998). For the present study, as the RFLP Southern method requires a large quantity of DNA, we developed the use of polymerase chain reaction PCR–RFLP markers in *B. vulgaris*. We investigated the joint cpDNA and mtDNA polymorphism over a large geographical area, covering Mediterranean regions of France and Spain, to assess the polymorphism of both genomes on such a geographical scale and to estimate the linkage disequilibrium value between the two organellar genomes. We draw conclusions on the mechanism (migration vs. mutation) by which the same mtDNA variant occurs over large distances and finally on the usefulness of both genomes for population genetics studies.

Materials and methods

Plant material

Beta vulgaris is a gynodioecious species that is widely distributed in Europe. Natural populations of sea beets (*B. vulgaris* ssp. *maritima*) are widespread along European coastlines. In the western part of the Mediterranean area, they are also present in man-disturbed inland habitats. In addition, cultivated beets (*B. vulgaris* ssp. *vulgaris*) are commonly found in several European countries. We

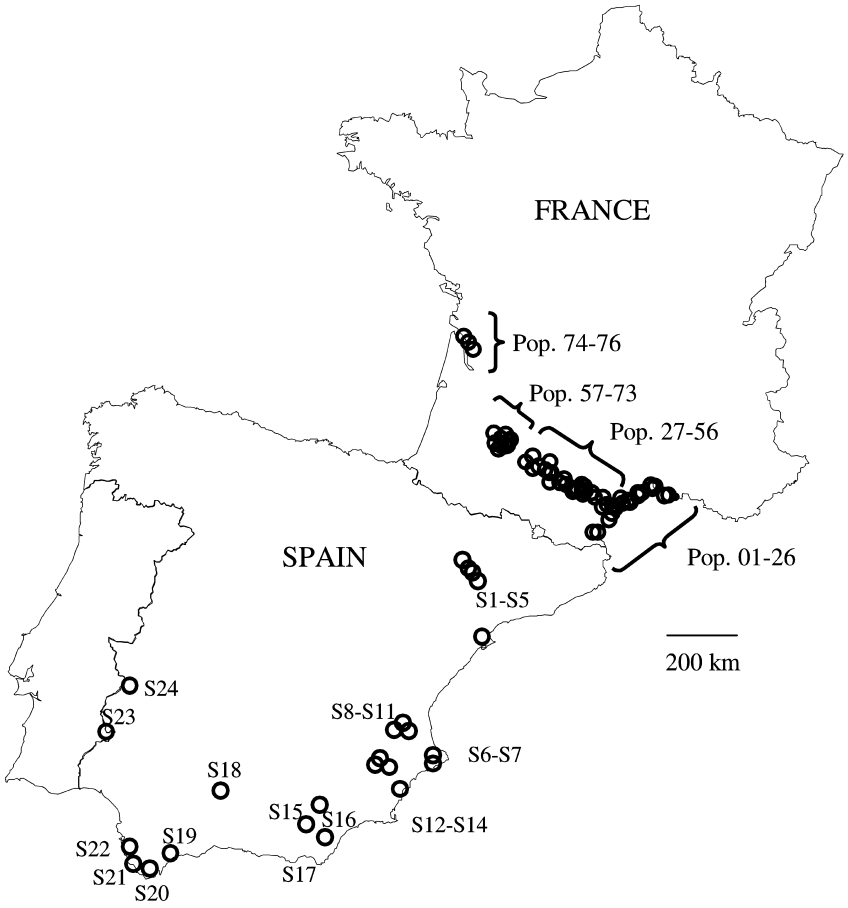

Fig. 1 Location of the natural populations (Pop.) sampled.

sampled 414 individuals of *B. vulgaris* ssp. *maritima* on a regional scale (Fig. 1, Appendix I) from the southern Mediterranean regions of France (293 plants from 76 populations) and Spain (121 plants from 24 populations). Plants were collected from different ecological situations (coastal and inland). The mean number of individuals sampled per population was 4.5 ± 0.2, as we chose to increase the number of populations rather than the number of plants per population (see a discussion about the sampling strategy in Pons & Petit 1995).

Laboratory analysis

Total genomic DNA was extracted from young leaves (1 g) following a modified Dellaporta procedure (as described in Saumitou-Laprade *et al*. 1991). The plants used were grown in a greenhouse from seeds collected from natural populations, except for 13 Spanish populations, for which fresh leaf material was collected in the field and dried over silica gel before DNA extraction. Each individual was analysed for both mtDNA and cpDNA polymorphism.

mtDNA polymorphism

The mitochondrial genome was investigated using RFLP (Southern blotting). For each individual sample, ≈ 5 µg of DNA was digested with *Eco*RI (2 U/µg). DNA fragments were separated on 0.7% agarose gels in TAE buffer (40 mM Tris-acetate, pH8, 1mM EDTA), and blotted onto a Nylon membrane (Allefs *et al*. 1990). Three mitochondrial probes were used: ATP6 (the ATPase subunit 6 from maize); pBV4 (a noncoding mitochondrial sequence from sugar beet); and NvulgN2 (a 12.5-kb fragment isolated from *Eco*RI digestion of mtDNA of beets). Probes were labelled, hybridized with total DNA and detected as described in Cuguen *et al*. (1994). Polymorphic phenotypes were then determined by analysis of the *Eco*RI restriction fragments. Mitotypes (i.e. mitochondrial haplotypes) were subsequently determined according to the phenotypes obtained with the three probes and named from A to U, plus Nvulg and Svulg (Table 1), using the same nomenclature as described in Cuguen *et al*. (1994).

cpDNA polymorphism

Recent studies demonstrated, for many plants, the usefulness of universal primers for assessing chloroplast polymorphism among populations of a given species (see for example Demesure *et al*. 1996; El Mousadik & Petit 1996a; Dumolin-Lapegue *et al*. 1997b; King & Ferris 1998;

Table 1 Definition of the mitochondrial DNA (mtDNA) haplotypes

	Hybridization pattern		
Mitotype	*Eco*RI/ATP6	*Eco*RI/pBV4	*Eco*RI/NvulgN2
A	2	2	1
B	2	3	2
C*	2	4	3
D*	2	5	1
E	1	2	4
F	3	3	2
G	2	1	5
H	2	3	6
I	2	3	3
J*	2	4	8
K	2	3	1
L	2	5	8
M	3	3	1
N	3	3	6
Nvulg	2	4	1
O	3	3	3
P	1	3	4
Q	3	5	4
R	3	7	9
S	2	3	9
Svulg	1	6	7
T	4	6	7
U	2	8	1

Each mitotype is defined by the combination of variants observed by using three probes (see the Materials and methods for details).
*C, D and J were not observed in the present data set but occur in beets from other French areas (Cuguen *et al*. 1994).
Length of the discriminant fragments of each variant (the hybridization pattern, in boldface) is followed by the size (in kbp). *Eco*RI/ATP6: **1**, 3.0; **2**, 4.0; **3**, 3.2; **4**, 2.5. *Eco*RI/pBV4: **1**, 3.0; **2**, 3.7, 3.0; **3**, 4.4, 3.0; **4**, 7.2, 3.0; **5**, 4.4; **6**, 5.1; **7**, 4.4, 2.6; **8**, 4.6, 3.0. *Eco*RI/NvulgN2: **1**, 15.1, 12.5, 10.9, 10.2; **2**, 15.1, 10.9, 10.2, 8.3; **3**, 15.1, 10.9, 10.2, 9.6; **4**, 15.1, 10.9, 10.2; **5**, 15.1, 10.2; **6**, 15.1, 13.2, < 10.9, 10.2; **7**, 15.1, < 10.9, 10.2; **8**, 15.1, > 10.9, 10.9, 10.2; **9**, 15.1, 15.0, 10.9, 10.2.

Marchelli *et al*. 1998). These universal primers are located in coding regions of cpDNA, separated by potentially more variable noncoding regions (mostly intergenic regions, see Demesure *et al*. 1995; Dumolin-Lapegue *et al*. 1997a). We applied this method for the first time in *B. vulgaris*. A set of 15 pairs of primers was used for a first screening of 21 plants. Seven pairs of primers were chosen because of their good amplification pattern and polymorphism. An additional primer pair (hereafter referred to as locus *X*), specific for *B. vulgaris*, was used, as this specific primer pair is known to distinguish the cytoplasm associated with Owen's male sterility (commonly found in cultivated European sugar beets) from cytoplasm of wild accessions (Ran & Michaelis 1995). PCR amplifications were performed in a DNA thermal cycler (Perkin Elmer). Reactions were carried out in a total volume of 15 μL using PCR buffer (1×), $MgCl_2$ (3.5 mM), dNTPs (100 μM), primers (2 pmol), DNA polymerase (0.5 U), bovine serum albumin (0.2 μg/μL) and genomic DNA (≈ 25 ng). An initial 5-min denaturation at 94 °C was followed by 30 cycles of 94 °C for 45 s, annealing for 45 s and elongation at 72 °C for 2–4 min. Annealing temperature was dependent upon the primers used and the extension time was dependent upon the length of the PCR product (see Demesure *et al*. 1995; Dumolin-Lapegue *et al*. 1997a). A final elongation for 10 min at 72 °C was performed. PCR products (5 μL) were digested with a 4-bp cutting restriction enzyme, *Hin*fI or *Alu*I (Table 2), for 2 h at 37 °C. Restriction digests were separated by electrophoresis in 8–10% polyacrylamide gels using Tris-borate EDTA buffer (0.5×) at 100 V for 19–24 h and visualized by silver staining. The size of the bands associated with each variant were scored according to the measurements obtained using the DENSYLAB™ software (Bioprobe™ Systems). More precisely, to assess size differences as small as 1 bp on 10% acrylamide gels, we chose an appropriate molecular weight marker (marker X from Boehringer Mannheim). After a first measure of the absolute size, the different variants for each polymorphic locus were rescreened on the same gel to ascertain the relative size differences. For locus *X*, amplifications were carried out in a total volume of 15 μL according to the method described by Ran & Michaelis (1995). PCR products were digested with *Hin*dIII and restriction digests were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. cpDNA variants at each locus were numbered in order of decreasing molecular weight. The length fragments were recorded from 1 to 5, 9 being reserved for the occurrence of a restriction site (Table 2). The numbers increase from the highest to the lowest molecular weight fragments to simplify the notation but this does not imply any sequence of mutational events.

Statistical analysis

Haplotype diversity (expected heterozygosity, H_e) values were calculated according to Nei (1987). The association (hereafter referred to as cytotype) between one mitotype and one chlorotype was designated by the mitotype code followed by the chloroplast code (for example: Nvulg-V is the combination of the mitotype Nvulg and the chlorotype V). Linkage disequilibrium between the two organellar genomes was calculated according to Lewontin's normalized D′ (Lewontin 1964; see Lewontin 1988 for a discussion on gametic disequilibrium) by using the ARLEQUIN software (Schneider *et al*. 1997).

Table 2 Cytoplasmic DNA (cpDNA) variants

Primers	Primer code	Size of PCR product (bp)	Enzyme	Variants					
				1	2	3	4	5	9
PsaA-trnS*	AS_2	3260	*Hin*fI	232					125 + 107
TrnH-trnK1*	HK	1520	*Alu*I	346					232 + 114
X87637†	X	563	*Hin*dIII	563					454 + 109
PsaA-trnS*	AS_1	3260	*Hin*fI	346	328				
TrnT-psbC‡	TC	3380	*Hin*fI	240					
TrnS-trnM1*	SfM	1220	*Hin*fI	148	144				
TrnD-trnT*	DT	970	*Hin*fI	98	97				
TrnK2-trnQ‡	KQ	2700	*Hin*fI	94	93	91			
TrnV2-rbcl2‡	VL	3700	*Hin*fI	113	112	110	108	105	

The total length of the polymerase chain reaction (PCR) product is given together with a description of the different variants observed for each primer pair and locus. The size of the bands associated with each variant is given according to the measurments obtained with the DENSYLAB™ software (Bioprobe™ Systems).
*Demesure *et al*. (1995); †Ran & Michaelis (1995); ‡Dumolin-Lapegue *et al*. (1997).

Results

Extent of cytoplasmic variation

mtDNA and cpDNA haplotypes The mtDNA and cpDNA haplotypes found in each population and the mean number of haplotypes per population are detailed in Appendix I. For the mitochondrial genome, 20 mitotypes were observed over the whole sample (the RFLP fragment patterns according to the *Eco*RI/mtDNA probes combinations are displayed in Table 1). All the mitotypes were widely distributed (Fig. 2), except the mitotypes L, O, P, Q, S and U, which were limited to a single population. The haplotype diversity for mtDNA markers was 0.65 ± 0.02. Despite the limited number of individuals analysed per population (4.5 ± 0.2), 53% of the populations (having more than two individuals sampled) exhibited two or more mitotypes.

The number of cpDNA variants found for each primer pair is given in Table 2. Primer pair TC exhibited only one variant from the present data set, although this primer pair was known to be polymorphic from preliminary studies involving populations from northern France. All the other primer pairs exhibited only one polymorphic locus, except for AS, which had two loci. Three of these displayed a restriction site mutation. The others were assumed to be insertions or deletions (indels). The size of the bands associated with these indels is given in Table 2: they ranged from 4 to 18 bp except for the loci DT, KQ and VL. These three cpDNA loci exhibited a different mutational pattern. More precisely, they were characterized by small indels (less than 4 bp) and/or by a larger number of variants (five variants for VL). Such a pattern may be the result of polymorphism of long stretches of mononucleotides A/T, as has been observed in other species (e.g. Gielly & Taberlet 1994). Owing to a different mutational rate when compared with restriction sites or larger indels, the results we obtained using these loci should be interpreted with caution. Thirteen haplotypes were detected over the whole study area (Table 3, Fig. 2). Chlorotypes I and XII were the only ones to be restricted to a single population. The chlorotype diversity value was 0.63 ± 0.03, not significantly different from the mitotype diversity. Here again, more than 50% (actual value 54%) of the populations exhibited two or more chlorotypes (when more than two individuals were sampled).

Association between mtDNA and cpDNA haplotypes

Twenty-six associations (cytotypes) between chlorotypes and mitotypes were observed (upper part of Table 4). The mean number of mtDNA types per cpDNA type was 1.86 ± 0.69. The mean number of cpDNA types per mtDNA type was 1.29 ± 0.27. Four mitotypes out of 20 (namely A, G, I and Nvulg) were associated with several chlorotypes. We also conducted an analysis of the association without the three loci DT, KQ and VL, owing to their aforementioned particularity. When chlorotypes were defined without the contribution of these loci, the number of chlorotypes changed into six (Table 3) and the number of cytotypes decreased to 21. Consequently, only mitotype I was found to be associated with more than one chlorotype (Table 4, lower part). The mean number of mtDNA types per cpDNA types then reached 3.14 ± 1.72, with 1.05 ± 0.10 cpDNA types per mtDNA. A strong linkage disequilibrium between the two organellar genomes was found. The D′ value was 0.965 when all the chloroplast loci were taken into account and reached 0.995 when DT, KQ and VL were excluded. Variation at the locus SfM was characterized by only two alleles that were separated

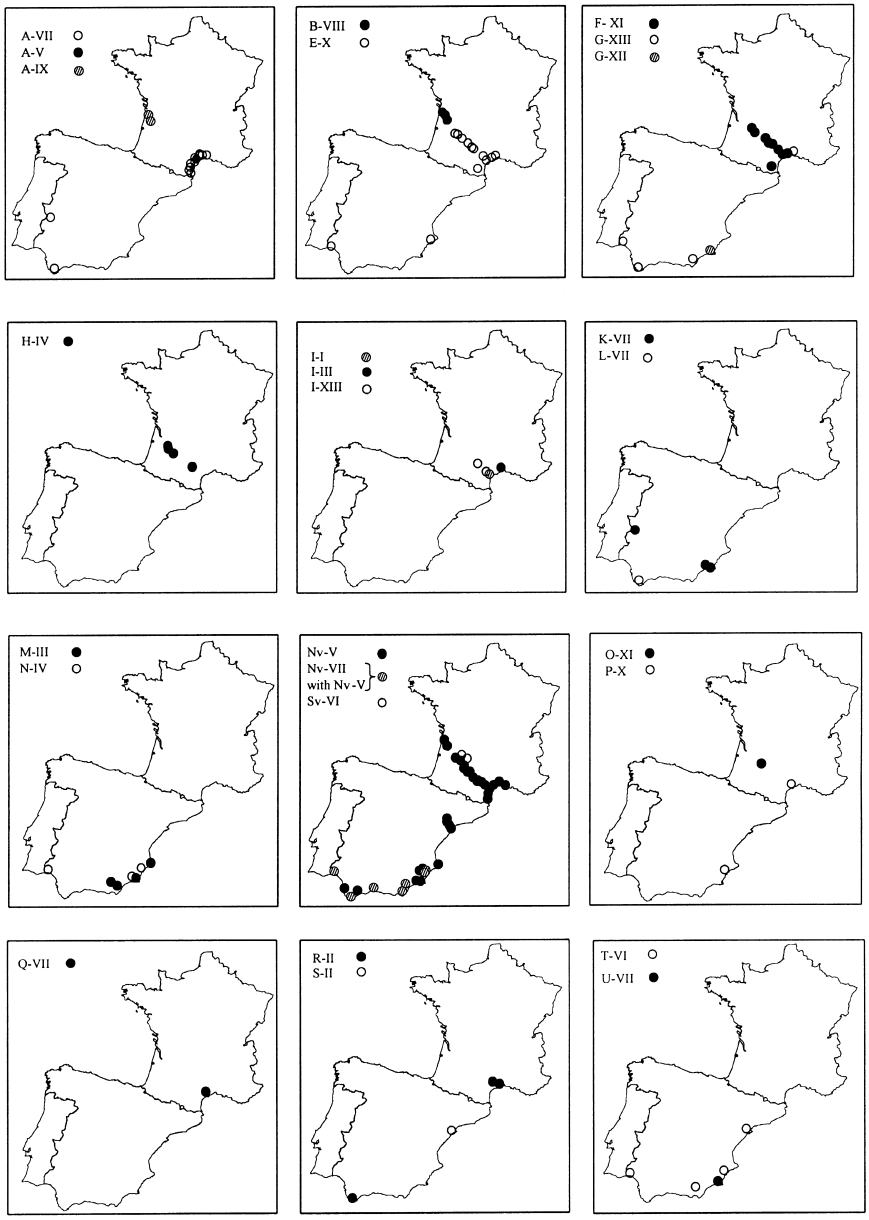

Fig. 2 Geographical location of the different associations between mitochondrial DNA and chloroplast DNA haplotypes observed in beets. The association between one mitotype and one chlorotype is designated by the mitotype code followed by the chloroplast code. Nv, Nvulg; Sv, Svulg.

by 4 bp. Such a pattern could perhaps also be caused by a microsatellite repeat, although a smaller size difference or a larger number of variants would be expected at a within-species level. When SfM was also removed from the analysis, chlorotypes Ibis and IIbis were pooled together but this did not change the results observed when only DT, KQ and VL were removed (in particular, mitotype I was still associated with two chlorotypes).

Discussion

mtDNA diversity

From our study, 20 mitotypes have been observed among 414 individuals sampled from widely distributed populations (1000 km between the most remote populations). This level of polymorphism is in agreement with previous studies on higher plant mitochondria. The mtDNA genome is known to exhibit extraordinary plasticity (Fauron *et al*. 1995), generating high levels of polymorphism. In *Thymus vulgaris*, for example, more than 50 mitotypes have been found among ≈ 400 individuals (Belhassen *et al*. 1993; Manicacci *et al*. 1996). In *Daucus carota* ssp. *carota*, 25 mtDNA RFLP variants were identified from 80 plants (Ronfort *et al*. 1995). In *Hevea brasiliensis*, 212 mtDNA RFLP variants were revealed in a sample of 395 accessions (Luo *et al*. 1995). A high mitochondrial polymorphism has also been found in three *Pinus* species (Strauss *et al*. 1993).

Table 3 Chloroplast DNA (cpDNA) haplotypes for *Beta vulgaris*

Haplotype code	Haplotype code bis	Primer code								
		AS_2	HK	X	AS1	TC	SfM	DT	KQ	VL
I	VI bis	9	1	1	2	1	1	1	3	4
II	I bis	9	1	1	1	1	1	2	2	1
III	I bis	9	1	1	1	1	1	2	2	2
IV	I bis	9	1	1	1	1	1	2	3	5
V	II bis	9	1	1	1	1	2	2	3	2
VI	III bis	9	1	9	1	1	2	2	3	2
VII	II bis	9	1	1	1	1	2	2	3	3
VIII	II bis	9	1	1	1	1	2	2	3	4
IX	II bis	9	1	1	1	1	2	2	3	5
X	IV bis	9	9	1	1	1	2	2	3	2
XI	V bis	1	1	1	2	1	1	2	2	4
XII	VI bis	9	1	1	2	1	1	2	2	4
XIII	VI bis	9	1	1	2	1	1	2	3	4

Each cpDNA haplotype is defined by the combination of variants obtained with nine loci and using eight primer pairs (see the Materials and methods for details).
Haplotype code 'bis' refers to the definition of a haplotype without primers DT, KQ and VL (see the Results for details).

Table 4 Association between chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) haplotypes (number of occurrences): the chlorotypes were defined with all cpDNA loci (upper part of the table) or without the cpDNA loci DT, KQ and VL (lower part of the table; see text for details). Nv stands for Nvulg and Sv for Svulg, respectively

	mtDNA																				
cpDNA	H	N	Sv	T	E	P	R	S	F	O	B	M	K	L	Q	U	Nv	A	G	I	Total
II							3	6													9
IV	10	5																			15
XI									30	1											31
VIII											8										8
VI			2	8																	10
X					26	2															28
V																	233	2			235
VII													6	1	1	2	14	26			50
IX																		2			2
XII																			1		1
III												7								3	10
I																				3	3
XIII																			10	2	12
Total	10	5	2	8	26	2	3	6	30	1	8	7	6	1	1	2	247	30	11	8	414
IIIbis			2	8																	10
IVbis					26	2															28
Vbis									30	1											31
IIbis											8		6	1	1	2	247	30			295
Ibis	10	5					3	6				7								3	34
VIbis																			11	5	16
Total	10	5	2	8	26	2	3	6	30	1	8	7	6	1	1	2	247	30	11	8	414

From the literature, the distribution of the mitochondrial genetic diversity does not exhibit a common feature. On the one hand, the DNA mitochondrial polymorphism appears to be geographically highly structured at a local scale (e.g. *T. vulgaris* in Manicacci *et al*. 1996) or a regional scale (*H. brasiliensis* in Luo *et al*. 1995, e.g. *D. carota* ssp. *carota* in Ronfort *et al*. 1995; *F. crenata* in Tomaru *et al*. 1998; *Abies* sp. in Tsumura & Suyama 1998).

On the other hand, large geographical distributions of some ubiquitous mitochondrial haplotypes may also be found (*Theobroma cacao* in Laurent *et al*. 1993, e.g. *F. crenata* in Koike *et al*. 1998; *Glycine soja* in Tozuka *et al*. 1998). A widespread distribution of haplotypes was also observed in *Beta vulgaris* ssp. *maritima* in a previous study carried out by Cuguen *et al*. (1994). By using a sample of 200 plants collected from 38 French populations, it was observed that most of the 14 mitochondrial RFLP patterns were ubiquitous at the scale studied and that the mitotype Nvulg (shared by 23% of the individuals) was widely distributed. Finally, many cases of species exhibiting frequent mtDNA variants distributed over a large geographical repartition were reported, suggesting that recurrent mutations could be suspected. Indeed, in three *Pinus* species, a phylogenetic study revealed, together with a high mitochondrial polymorphism, frequent homoplasic events (Strauss *et al*. 1993).

The structure of the mitochondrial genome is actually represented as a population of small circular molecules (subcircles) created from a master circle through intragenomic recombination (Andre *et al*. 1992; Palmer 1992; Fauron *et al*. 1995). As the substitution rate of nucleotides is very low in this genome (Wolfe *et al*. 1987; Palmer & Herbon 1988), differences between mtDNA restriction profiles within the same species are more likely to be caused by rearrangements than by point mutations. The genome seems therefore to evolve mainly by intragenomic recombination through small repeated sequences dispersed within the genome (Lonsdale *et al*. 1988, e.g. in Lelandais *et al*. 1998). In *B. vulgaris*, the mitochondrial genome is known to include short dispersed repeated sequences through which intragenomic recombination is thought to occur (see Senda *et al*. 1998a for an example). Kubo *et al*. (1995) have shown that higher order repeats could be found within the mitochondrial genome associated with the mitotype Nvulg of *B. vulgaris*. The hypothesis of recurrent appearance of a given mtDNA genotype in different populations therefore cannot be rejected only from the examination of mitochondrial RFLP phenotypes.

From the present study, nine mitotypes out of the 20 observed were reported for the first time. The mitotypes F, K and L, which were rarely found by Cuguen *et al*. (1994), were found at a higher frequency in the present study, but the mitotypes C, D and J were not observed in the area studied. Conversely, some new, rare mitotypes were found (for example O, P, Q, S and U). In agreement with previous studies in beets (Saumitou-Laprade *et al*. 1993; Cuguen *et al*. 1994), as well as in other species, it appears that the same mitotypes can be found over a large geographical scale in *B. vulgaris*. In addition, the populations usually exhibited more than one mtDNA haplotype, even when a small number of individuals was sampled. For these reasons, recurrent mutations in *B. vulgaris* cannot be *a priori* excluded to explain the geographical distribution of mitotypes in beets.

cpDNA diversity

We identified 13 cpDNA haplotypes, three of which involved a point mutation in a restriction site, the others being assumed to be indels. The chloroplast genome is known to evolve mainly through point mutations or small deletions (Clegg *et al*. 1992). Clegg *et al*. (1994) reported that the frequency of indels was higher than that of point mutations. The patterns of polymorphism presented here, as well as in other studies using PCR–RFLP methods (e.g. Demesure *et al*. 1996; Dumolin-Lapegue *et al*. 1997b; Petit *et al*. 1997) are in agreement with that report (nevertheless, technical approaches used here have a higher probability of revealing indels than point mutations, owing to the low number of restriction enzymes used).

Investigations of other species conducted using the same sampling and molecular tools (i.e. a screening of 100–450 individuals at a regional scale, using six to 12 universal primer pairs and one to eight restriction enzymes) revealed a comparable number of haplotypes. For example, 11 haplotypes were found in *Fagus* (Demesure *et al*. 1996), 13 in *Alnus* (King & Ferris 1998), 11 in *Argania* (El Mousadik & Petit 1996a) and up to 23 haplotypes in the white oaks species complex (Dumolin-Lapegue *et al*. 1997b), but over a larger geographical scale. From our results, it appeared that cpDNA diversity can be efficiently investigated in beets by PCR–RFLP using universal primers. Most of the species mentioned above also showed a strong regional differentiation of cpDNA haplotypes (Demesure *et al*. 1996; El Mousadik & Petit 1996b; Dumolin-Lapegue *et al*. 1997b; Wolf *et al*. 1997; King & Ferris 1998; Marchelli *et al*. 1998) or a patchy structure when investigated within a region (Petit *et al*. 1997). In *B. vulgaris*, three haplotypes out of 13 were each restricted to one or two populations. The other haplotypes were found both in France and Spain, with no obvious geographical structure, which contrasts with species where cpDNA haplotypes are geographically localized, even at a finer scale (Wolf *et al*. 1997; King & Ferris 1998; Marchelli *et al*. 1998). Populations of beets were found to be far from fixation for their chlorotypes and exhibited 1.74 ± 0.17 chlorotypes per population. These results are in close agreement with those previously reported by Forcioli *et al*. (1998) in a study of the cpDNA diversity of the French populations of beets from the Atlantic and Channel coasts, using a similar sampling strategy but involving a Southern RFLP analysis: eight haplotypes were found, 80% of the populations appeared polymorphic, with a mean number of haplotypes of 2.17 ± 0.66. We can therefore conclude that there is a substantial within-

population diversity in *B. vulgaris* ssp. *maritima* as shown previously in several other species (McCauley 1994; El Mousadik & Petit 1996a; Raspe 1998), although it is not a general feature (Demesure *et al*. 1996; Marchelli *et al*. 1998).

The association between cpDNA and mtDNA haplotypes

The chloroplast and the mitochondrial genomes are both maternally transmitted in beets (Corriveau & Coleman 1988), as it is the case for the majority of angiosperms (Reboud & Zeyl 1994). Very high values of haplotypic disequilibrium were observed between the cpDNA and mtDNA genomes (D′ = 0.97 by using all the cpDNA loci and D′ = 0.99 without DT, KQ and VL). Our results are in agreement with the expectation of a maximum linkage between the two organellar genomes, owing to their co-transmission. The linkage value is expected to be weakened: (i) if some paternal transmission takes place (Moggensen 1996); or (ii) if recurrent mutations occur in one or both genomes.

The number of individuals of a single progeny that have to be investigated to detect paternal leakage is high (Milligan 1992). In the present study, the sampling over progeny and populations was not well designed for this purpose. Nevertheless, the high value of the disequilibrium estimate is not in agreement with a frequent reshuffling of both genomes. By investigating the occurrence of paternal leakage of cpDNA or mtDNA in oaks, Dumolin-Lapegue *et al*. (1998) observed nine cpDNA and three mtDNA variants, which defined 12 cytotypes. The strong association observed between the two cytoplasmic polymorphisms suggested no well-established paternal leakage. The partial independence between the two genomes was caused only by one mtDNA haplotype. They suggested that this haplotype may have arisen from recurrent homoplasic mutation.

The observed linkage disequilibrium value estimated from the present study was also inconsistent with a high rate of homoplasic recombination of mtDNA in beets. For that reason, the wide geographical distribution of a particular mitotype, as we observed in *B. vulgaris*, will probably be a result of migration. This finding does not contradict the models of mitochondrial evolution through recombination and frequency shifts (Atlan & Couvet 1993) but seems to exclude frequent homoplasic mutations. The few cases of departure from a strict association have to be carefully examined. Some mitotypes (A, G, I and Nvulg) were found to be associated with up to three chlorotypes. This phenomenon was mainly caused by three cpDNA loci (DT, KQ and VL), which exhibited very small indels. It totally disappeared (except for mitotype I) when DT, KQ and VL were excluded from the definition of the chlorotypes. For each of the mitotypes A, G and Nvulg, a given chlorotype (VII, XIII and V, respectively) was always predominant, together with one or two other rare chlorotypes. The geographical distribution of unusual associations was clearly not random (see Fig. 2). For example, we found three cytotypes involving mitotype A in 30 individuals from 14 French and Spanish populations. Twenty-six were characterized by the widely distributed cytotype A-VII. Two individuals from one single French Mediterranean population showed cytotype A-V and, finally, two individuals who exhibited cytotype A-IX were restricted to two adjacent populations of the Atlantic coast. The same feature was observed for the cytotypes involving mitotypes G. Another interesting obsevation is that, except for mitotype Nvulg, there were no populations characterized by several individuals sharing the same mitotype but different chlorotypes. Out of 100 populations analysed, 84 populations exhibited individuals with the mitotype Nvulg. In all of those populations Nvulg was found to be associated with the chlorotype V. However, in seven (out of 84) populations from Spain, Nvulg was also found to be associated with chlorotype VII (see Fig. 2). Chlorotypes V and VII differed only by a small indel at locus VL. Such small indels can be caused by mononucleotide repeats. Such stretches are commonly found in the cpDNA genome and have been increasingly used in the past few years for population biology studies (Powell *et al*. 1995; Vendramin *et al*. 1996; Echt *et al*. 1998; Provan *et al*. 1998, 1999; Vendramin *et al*. 1998). The mutation rate of such repeats is substantially higher than for other anonymous regions (Powell *et al*. 1995) and could explain the high variability that we observed at locus VL compared with the other loci used. This may also hold for the loci DT and KQ, for which indels of less than 4 bp were observed. Sequencing of the variants that differ by few base pairs has to be conducted in order to precisely determine the type and number of mutations involved. If the implication of microsatellite loci is confirmed, the high level of polymorphism exhibited could be of great interest for further population genetics studies at a fine geographical scale in wild beets.

Events of recurrent recombination in the mitochondrial genome could not always be excluded in our study. This was the case for mitotype I, which was associated with three chlorotypes, at the same frequency, when using nine loci for defining the chlorotypes. It still presented two chlorotypes when the loci DT, KQ and VL were excluded from the analysis.

The strong association observed here may also give some insights into the relatedness between mitotypes. In a previous study, Cuguen *et al*. (1994) discussed the correlation between mitotype and cytoplasmic male sterility in gynodioecious populations of *B. vulgaris* ssp. *maritima*: four mitotypes (Svulg, E, G and H) out of 14 were found to be associated with male sterility. Sugar beets are

characterized by the mitotype Svulg (Senda *et al*. 1998b) and the chlorotype VI (unpublished data; present study). Here, we observed a new mitotype, T, also associated with chlorotype VI. The observation that Svulg and T share a common chlorotype may indicate that both mitotypes are closely related. The same feature holds for mitotypes P and N, which share the same chlorotype as E and H, respectively. The sexual phenotypes associated with mitotypes T, P and N have not been previously observed and further investigations obviously need to be carried out.

Finally, the restricted association of mitotype Svulg with chlorotype VI and, more specifically, with variant 9 of primer *X*, is of special interest for investigating the presence of Svulg in natural populations. Because mitotype screening is laborious and requires a large quantity of DNA, the use of the primer *X* may be useful for the rapid detection of Svulg in natural populations. Once chlorotype VI has been identified, a limited mtDNA RFLP analysis has to be conducted to check that Svulg is involved, as this cpDNA pattern is shared by the rare mitotype T, only observed in Spain. The occurrence of Svulg in the wild can be used as an indicator of seed dispersal from crops to wild populations. Such investigation can be of great value in the context of risk assessment of genetically modified organisms (GMOs).

Conclusion

Cytoplasmic markers are now commonly used by population geneticists. Their application, to estimate the relative importance of seed and pollen dispersal within or among populations, is now widespread (Cruzan 1998). However, contrary to cpDNA variation, mtDNA polymorphism has frequently been viewed with caution when studying plant population differentiation. In this study, we demonstrated the occurrence of a particularly strong association between the two cytoplasmic haplotypes in beets. In only a few cases, at a large geographical scale, a given mitotype was not associated with a single chlorotype. The geographical distribution of such peculiar cases was clearly not random. Our results strongly suggest that mutations in the cpDNA will probably be involved in these cases, and that recurrent mitochondrial mutations could be excluded. Consequently, it appears that distribution of the mtDNA haplotypes in *B. vulgaris* can be mainly explained by migration. Seed migration in beets at various geographical scales is mainly caused by tides, for coastal populations, and human activities (e.g. roadworks), for inland populations (Van Dijk & Desplanque, in press). We conclude that mitotypes could be as valuable as chlorotypes when investigating population genetic structure, phylogeographical patterns or seed dispersal in natural populations.

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One of the research programmes of the laboratory 'Génétique et Evolution des Populations Végétales' is currently investigating the evolution of the reproductive system and life history traits in the genus *Beta*. B. Desplanque is a PhD student in evolutionary genetics under the supervision of H. Van Dijk, with particular interest in the relationships between wild and cultivated beets. For the present study, his work was also supervised by F. Viard, a CNRS researcher involved in the study of population genetics, reproductive systems and population dynamics in *Beta* species.

Appendix I Mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) haplotypes for the wild populations of *Beta vulgaris* ssp. *maritima* studied

		A			Haplotype code (number of haplotypes)	
Code	Name of the population	*n*	mtDNA	cpDNA	mtDNA	cpDNA
1	Stes Maries de la mer	4	1	1	Nv (4)	V(4)
2	La Grande-Motte	4	1	1	Nv (4)	V (4)
3	Palavas les flots	4	1	2	Nv (4)	V (3), VII (1)
4	Maurin	3	2	2	A (1), Nv (2)	V (1), VII (2)
5	Lattes	6	2	2	G (4), Nv (2)	V (2), XIII (4)
6	Villeneuve les Maguelones	5	4	3	A (1), E (2), Nv (1), R (1)	II (1), V (3), VII (1)
7	St Jean de Vedas (1)	3	1	1	A (3)	VII (3)
8	St Jean de Vedas (2)	3	1	1	I (3)	III (3)
9	Balaruc les Bains	4	1	1	Nv (4)	V (4)
10	Meze (1)	5	2	2	A (3), Nv (2)	V (2), VII (3)
11	Meze (2)	5	2	2	A (4), Nv (1)	V (1), VII (4)
12	Meze (3)	3	2	2	Nv (2), Q (1)	V (1), VII (2)
13	Sete	2	2	2	E (1), Nv (1)	V (1), X (1)
14	Marseillan	5	3	3	A (2), F (1), Nv (2)	V (2), VII (2), XI (1)
15	Agde	2	1	1	A (2)	V (2)
16	Bezier	5	2	2	Nv (4), R (1)	V (4), II (1)
17	Valras plage	4	2	2	A (3), Nv (1)	V (1), VII (3)
18	St Pierres/Mer	5	2	2	E (1), Nv (4)	V (4), IX (1)
19	Bages	4	2	2	A (2), Nv (2)	V (2), VII (2)
20	Fleury	4	1	1	Nv (4)	V (4)
21	Gruissan	5	2	2	F (4), Nv (1)	V (1), XI (4)
22	Leucate	5	2	2	A (4), Nv (1)	V (1), VII (4)
23	Toulouge	5	1	1	Nv (5)	V (5)
24	Millas	4	1	1	E (4)	X (4)
25	Portel les Corbières	5	2	2	F (3), Nv (2)	V (2), XI (3)
26	Ferrals	5	2	2	I (3), Nv (2)	I (3), V (2)
27	Conhitac	4	1	1	Nv (4)	V (4)
28	Marseillette	2	1	1	Nv (2)	V (2)
29	Conques/Orbiel (1)	4	2	2	E (2), Nv (2)	V (2), X (2)
30	Conques/Orbiel (2)	5	3	3	F (3), I (1), Nv (1)	V (1), XI (3), XII (1)
31	Carcassone (1)	3	1	1	Nv (3)	V (3)
32	Carcassone (2)	4	1	1	F (4)	XI (4)
33	Lastour	2	1	1	Nv (2)	V (2)
34	Villegailhenc	4	1	1	Nv (4)	V (4)
35	Alzonne	2	1	1	Nv (2)	V (2)
36	Prouille	4	1	1	Nv (4)	V (4)
37	Bram	4	3	3	H (1), I (1), Nv (2)	IV (1), V (2), XIII (1)
38	Villepinte (1)	5	1	1	F (5)	VI (5)
39	Villepinte (2)	5	1	1	Nv (5)	V (5)
40	Villeneuve la Comptal	3	1	1	F (3)	XI (3)
41	Estambigou	3	1	1	Nv (3)	V (3)
42	Castelnaudary (1)	3	1	1	Nv (3)	V (3)
43	Castelnaudary (2)	1	1	1	Nv (1)	V (1)
44	Montmaur (1)	3	2	2	F (1), Nv (2)	V (2), XI (1)
45	Montmaur (2)	3	1	1	Nv (3)	V (3)
46	Avignonnet en Lauragais	5	2	2	E (4), F (1)	X (4), XII (1)
47	Cintegabelle	4	1	1	Nv (4)	V (4)
48	Baziège	3	2	2	E (2), Nv (1)	V (1), X (2)
49	Lagardelles/Ceze	5	2	2	F (2), Nv (3)	V (3), XI (2)
50	Isle	1	1	1	Nv (1)	V (1)
51	Verfeil	5	1	1	Nv (5)	V (5)
52	Castanet Tolosan	3	2	2	E (2), Nv (1)	V (1), X (2)
53	Plaisance du Touch	3	2	2	E (2), F (1)	X (2), XI (1)
54	St Lys	3	1	1	Nv (3)	V (3)
55	Grenades/Garonne	5	1	1	Nv (5)	V (5)
56	Montaigus/Save	5	3	2	H (1), Nv (3), O (1)	V (3), XI (2)
57	Mestre	1	1	1	H (1)	IV (1)

Appendix I *Continued*

		A			Haplotype code (number of haplotypes)	
Code	Name of the population	*n*	mtDNA	cpDNA	mtDNA	cpDNA
58	Montestruc	5	3	3	F (1), Nv (3), Sv (1)	V (3), VI (1), XI (1)
59	Fleurance	2	2	2	H (1), Sv (1)	IV (1), VI (1)
60	Merens	4	2	2	E (1), Nv (3)	V (3), X (1)
61	Lavardens (1)	3	1	1	Nv (3)	V (3)
62	Lavardens (2)	3	2	2	E (2), Nv (1)	V (1), X (2)
63	Lectoure (1)	5	1	1	Nv (5)	V (5)
64	Lectoure (2)	1	1	1	Nv (1)	V (1)
65	Castelnau d'Arbieu	4	2	2	H (2), Nv (2)	IV (2), V (2)
66	St Puy (1)	4	1	1	Nv (4)	V (4)
67	St Puy (2)	10	2	2	H (5), Nv (5)	IV (5), V (5)
68	La Romieu	4	1	1	Nv (4)	V (4)
69	Ayguetinte	3	1	1	Nv (3)	V (3)
70	Nerac	1	1	1	Nv (1)	V (1)
71	Vic Férensac	1	1	1	Nv (1)	V (1)
72	Condom (1)	9	1	1	Nv (9)	V (9)
73	Condom (2)	5	1	1	Nv (5)	V (5)
74	St Seurin d'Euzet	5	2	2	A (1), B (4)	IX (1), VIII (4)
75	Talmont	4	2	2	B (3), Nv (1)	V (1), VIII (3)
76	Suzac	4	3	3	A (1), B (1), Nv (2)	IX (1), V (2), VIII (1)
S1	Ell muntels	8	2	2	S (6), T (2)	II (6), VI (2)
S2	Raval de Christ	5	1	1	Nv (5)	V (5)
S3	Bitem	5	1	1	Nv (5)	V (5)
S4	Tivenys	4	1	1	Nv (4)	V (4)
S5	Benifallet	5	1	1	Nv (5)	V (5)
S6	Los Arenetes	5	1	1	Nv (5)	V (5)
S7	Benissa	5	2	2	M (4), Nv (1)	III (4), V (1)
S8	Paula del este	4	2	3	Nv (3), T (1)	V (2), VI (1), VII (1)
S9	Crevillente	4	2	3	Nv (3), P (1)	V (1), VII (2), X (1)
S10	Cox	5	2	2	N (1), Nv (4)	IV (1), V (4)
S11	Rafal	5	1	1	Nv (5)	V (5)
S12	Llamas de alburon	5	4	4	G (1), K (1), M (1), Nv (2)	III (1), V (2), VII (1), XII (1)
S13	Sierra Espuna	4	2	2	K (1), N (3)	IV (3), VII (1)
S14	La Hoya	5	3	3	E (1), Nv (2), U (2)	V (2), VII (2), X (I)
S15	Albox	5	2	2	M (1), T (4)	III (1), VI (4)
S16	Agua amarga	6	2	3	M (1), Nv (5)	III (1), VII (4), XIII (1)
S17	Cabo de gata	6	2	3	G (1), Nv (5)	V (1), VII (4), XIII (1)
S18	Almayate	5	1	2	Nv (5)	V (3), VII (2)
S19	Estepona	6	1	1	Nv (6)	V (6)
S20	Tarifa	4	3	2	A (2), Nv (1), R (1)	II (1), VII (3)
S21	Vega	5	2	2	G (4), L (1)	VII (1), XIII (4)
S22	Puerto real	4	1	1	Nv (4)	V (4)
S23	Cartaya	6	5	5	E (2), G (1), N (1), Nv (1), T (1)	IV (1), VI (1), VII (1), X (2), XIII (1)
S24	Elvas	5	2	1	A (1), K (4)	VII (5)
	Mean ± SE	4.10 ± 0.30	1.65 ± 0.16	1.67 ± 0.16		
	Mean* ± SE	4.50 ± 0.25	1.72* ± 0.17	1.74* ± 0.17		
	Total	414	20	13		

The codes are the same as those used in Fig. 1. *n*, sample size, A, number of haplotypes per population. Nv denotes for Nvulg and Sv, Svulg, respectively.

*Estimates based on populations having a sample size of three or more individuals.