Theor Appl Genet (2000) 100:1304–1310

A. Mohanty · J.P. Martín · I. Aguinagalde

Chloroplast DNA diversity within and among populations of the allotetraploid *Prunus spinosa* L.

Received: 20 September 1999 / Accepted: 10 November 1999

Abstract High chloroplast DNA (cpDNA) diversity was found within and among populations of *Prunus spinosa* sampled from seven European deciduous forests. A study of 12% of the total chloroplast genome detected 44 mutations, which were distributed over 24 haplotypes; four were common to two or more populations and the rest were unique haplotypes. The most-abundant and widely distributed haplotype was H2 (frequency = 41% approximately). Six of the seven populations were polymorphic. All of the six polymorphic populations had "private" haplotypes (frequency < 5%) in addition to common haplotypes. The UPGMA dendrogram demonstrated a correlation between populations and their geographical locations. The total diversity was high (h_T = 0.824) and a major portion of it was within populations (h_s = 0.663). The level of population subdivision for unordered alleles was low (G_{ST} = 19.5%) and for ordered alleles was lower (N_{ST} = 13.6%). No phylogeographic structure could be demonstrated in the present geographical scale. High polymorphism in the cpDNA of *P. spinosa* has to be considered carefully when planning phylogenetic studies involving this species.

Key words *Prunus spinosa* L. · cpDNA diversity · PCR-RFLP · Universal primers

Introduction

Prunus spinosa L., commonly known as sloe or blackthorn, is frequently found in European deciduous forests. This wild shrub is an allotetraploid, (2n = 4*x* = 32; Reynders-Aloisi and Grellet 1994), insect-pollinated species which frequently reproduces vegetatively; seed dispersal is mainly by birds and animals (Yeboah and Woodell 1987; Guitian et al. 1993). This shrub has wide range of environmental adaptability including resistance to cold, calcareous soils and drought. Considering that *P. spinosa* represents one of the possible ancestors of *Prunus domestica* (Watkins 1976, 1981) the above mentioned attributes are of particular interest for the improvement of rootstocks or varieties through interspecific hybridization. In Spain, *P. spinosa* has gained much importance as an economically important plant because of its fruits which contain volatile components (Fernández-García et al. 1998) rendering it suitable for flavouring the popular alcoholic drink "Pacharan."

Because of its highly conserved nature with lower mutation rates than the plant nuclear genome, the chloroplast genome (Wolfe et al. 1987) has limited use in speciation studies and the phylogenetic analysis of populations within a species (Palmer 1987). Several studies which throw light on the extent of intraspecific variation in chloroplast DNA (cpDNA) have been carried out in a number of species (reviewed in Soltis et al. 1992). Polymorphisms in cpDNA have been used in many investigations to determine the phylogeography or population diversity of a species (Levy et al. 1996; Van Dijk and Bakx-Schotman 1997; Jackson et al. 1999; Segraves et al. 1999). The range of diversity encountered in this genome varies between nil as in *Pennisetum glaucum* (Clegg et al. 1984; Gepts and Clegg 1989), low in *Lupinus texensis* (Banks and Birky 1985), European oaks (Petit et al. 1993) and with high polymorphisms in *Trifolium pratense* (Milligan 1991), *Dioscorea bulbifera* (Terauchi et al. 1991) and *Eucalyptus nitens* (Byrne and Moran 1994). All of these studies used tranditional restriction fragment length polymorphism of entire cpDNA, where the variants reflect gain or loss of restriction-site or length variations.

More recently, universal primers (Taberlet et al. 1991; Demesure et al. 1995; Dumolin et al. 1997b) are being used to amplify specific fragments of cpDNA followed

Communicated by P.M.A. Tigerstedt

A. Mohanty (✉) · J.P. Martín · I. Aguinagalde
Departamento de Biología Vegetal,
Escuela Técnica Superior de Ingenieros Agrónomos,
Universidad Politécnica de Madrid, Ciudad Universitaria s/n,
28040 Madrid, Spain
e-mail: jpmartin@bio.etsia.upm.es
Fax: +34-91-3365656

by digestion with restriction enzymes, thus revealing fragment length polymorphisms within the amplified fragment (Demesure et al. 1996; El Mousadik and Petit 1996; King and Ferris 1998). This PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method is a readily accessible laboratory technique which can evaluate large portions of the chloroplast genome in numerous individuals in a short time and at a lower cost. This technique may reveal a larger number of variations as at least 50% of cpDNA variations are attributable to small insertions and deletions (Gaut et al. 1993; Gielly and Taberlet 1994).

P. spinosa is a less-investigated species and there have been no studies for the assessment of its genetic (nuclear and/or cytoplasmic) variability. This is the first report of a study of intraspecific cpDNA variations in the genus *Prunus*. The present paper highlights the detection of a large number of polymorphisms in the cpDNA (using the PCR-RFLP technique) of the wild shrub *P. spinosa* collected from seven deciduous forests across Europe.

Materials and methods

Plant material

Seven populations of *P. spinosa* were sampled from deciduous forests across Europe (Table 1). The number of individuals in each population varied between five and 11. The distance between individuals in every population was at least 200 m. This was important in order to avoid collecting the clones since suckering is common in *P. spinosa*. Fresh leaves were collected in the field, frozen and transferred to the freezer at –80°C.

DNA extraction, amplification and digestion

DNA was extracted from frozen leaves following the protocol of Torres et al. (1993). Extracted DNA was quantified and a working solution of DNA (4 ng/µl) was made.

Preliminary amplification of cpDNA was done using 16 pairs of universal primers (see Table 2); all are described in Dumolin et al. (1997b). The amplifications were performed in 30 µl of a reaction mixture consisting of 0.2 µM of each primer, 200 µM of each of the four dNTPs, 2 mM $MgCl_2$, 0.5–1.0 U of EcoTaq DNA polymerase in the buffer provided by the manufacturer of the enzyme (ECOGEN, S.R.L.), and 12 ng of genomic DNA. The PCR-amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc.) with a heated lid, using an initial cycle of 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 45–62°C, and 2–5 min 30 s at 72°C (the annealing temperature and extension time depending on the length of the fragment to be amplified; Table 2), and finally a 10-min extension at 72°C.

Three restriction enzymes (*Alu*I, *Hin*fI and *Taq*I; Amersham) were used for digestion of the PCR product (8.5 µl/enzyme). The reaction mixture (20 µl) was incubated for 18 h with two units of *Alu*I, *Hin*fI or five units of *Taq*I. Restriction fragments were separated on 2.6% agarose gels in Tris-borate EDTA buffer (1×), run at 3 V/cm for 4 h, stained with ethidium bromide and visualized in UV light. The digests were also resolved on 8% polyacrylamide gels run in TBE buffer (1×) at 350 V for 4–8 h (the time depending on the size of the fragment to be resolved). These gels were silver stained (Bassam et al. 1991).

The size of the polymorphic bands was analyzed with Kodak Digital Science 1D Image Analysis Software. A 50-bp Ladder from Pharmacia was used as molecular-size marker.

Analysis of data

The HAPLODIV program (Pons and Petit 1995) was employed to calculate the frequency of the haplotypes, the estimation of the parameters of cpDNA diversity (h_T = total diversity, h_s = average intrapopulation diversity, and the G_{ST} = level of population subdivision of diversity using unordered alleles) and their standard errors. The level of population subdivision for ordered alleles (N_{ST}), v_T and v_S (the analogues of h_T and h_S), were calculated using the HAPLONST program (Pons and Petit 1996).

A matrix of frequencies of haplotypes in each population was used for generating a matrix of distances between populations, selecting the coefficient of "Euclidean distances squared." This latter matrix was employed to construct a dendrogram for assessing relatedness among the seven populations by the UPGMA method, using the NTSYS-pc version 1.6 package (Rohlf 1992).

The number of mutational differences between haplotypes was calculated to produce a minimum-length spanning-tree of haplotypes using the programs from NTSYS-pc (Rohlf 1992). This procedure is used to connect points (haplotypes) by direct links having the smallest possible total length (Prim 1957). Minimum spanning networks are alternatives to Wagner parsimony trees, but better convey the connections between haplotypes (Excoffier and Smouse 1994).

Results

Sixteen pairs of universal primers of cpDNA were initially screened to assess the degree of amplification and the size of the amplified fragment (Table 2). Based on the quality of amplification, five pairs of primers (HK, K1K2, *rpo*CC, CD and VL) were chosen for the present investigation. The total size of the amplified fragments used in this study represent approximately 12% of the total chloroplast genome (considering that cpDNA size in most *Prunus* spp. is approximately 140 kbp: Kaneko et al. 1986; Uematsu et al. 1991). The five amplified fragments were digested by three restriction enzymes and, of the 15 combinations, nine (HK-*Alu*I, *Hin*fI; K1K2-*Hin*fI,

Table 1 List, origin and number of individuals in the populations of *P. spinosa* studied

Population	Abbreviation	Origin	No. of individuals
Montejo de la Sierra	MS	Spain	10
Valdemorillo (slopes of Guadarrama)	VA	Spain	5
Chizé	CH	France	11
Parco Nazionale delle Foreste Casentinesi	FC	Italy	10
Kelheim	KE	Germany	10
Halltorps Hage	HH	Sweden	10
Glen Affric	GA	Scotland	6

Table 2 Preliminary results showing PCR conditions, size of amplified fragment and quality of amplification, using cpDNA universal primers

Abbreviation of cpDNA primers[a]	PCR conditions		Amplified fragment (bp)	Degree of amplification[b]
	Annealing temperature	Extension time		
HK	62°C	2 min 30 s	1700	++
K1K2	53.5°C	3 min	2650	++
K2Q	45°C	4 min	2700	+
QR	56.2°C	4 min	–	NA
*rpo*CC	45°C	5 min 30 s	4800	++
CD	55°C	4 min 30 s	3800	++
DT	52°C	2 min	1150	+
TC	50°C	4 min	–	NA
CS	55°C	3 min	1700	+
SfM	62°C	2 min	1200	+
fMA	45°C	5 min 30 s	< 6000	+
AS	55°C	4 min 30 s	3250	+
ST	50°C	2 min	–	NA
TF	50°C	2 min 30 s	–	NA
FV	50°C	4 min 30 s	–	NA
VL	55°C	4 min 30 s	3900	++

[a] Abbreviations are the same as in Dumolin et al. 1997b
[b] NA: no amplification; +: faint amplification; ++: good amplification

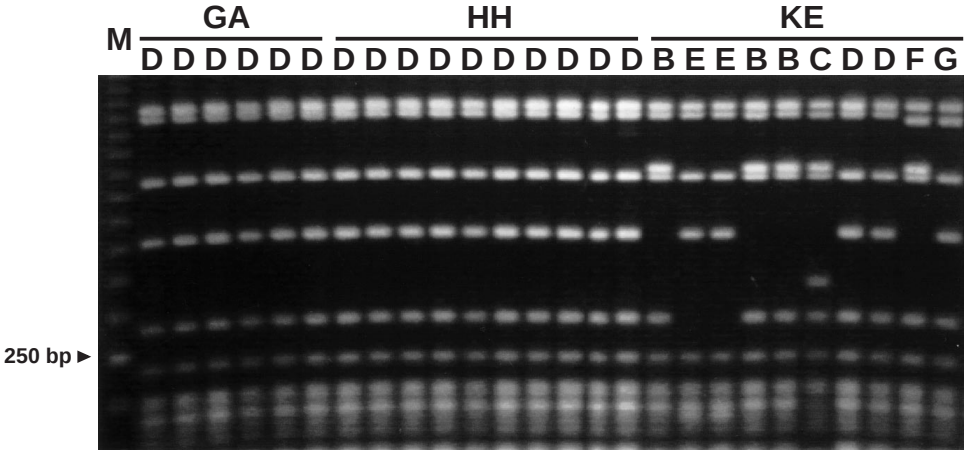


Fig. 1 Restriction patterns (**B**–**G**) in the primer-enzyme combination, *rpo*CC–*Hin*fI, of three populations (GA, HH, KE) on agarose gel. *B* to *G* correspond to the alphabets used in Appendix 1. *M* = molecular size marker (50-bp Ladder, Pharmacia). *KE* = Kelheim (Germany); *HH* = Hallotorps Hage (Sweden); *GA* = Glen Affric (Scotland)

*Taq*I; *rpo*CC-*Hin*fI; CD-*Alu*I, *Hin*fI, *Taq*I; VL-*Alu*I) exhibited polymorphic patterns and were hence selected for the complete survey (data can be obtained from the authors). The restriction patterns obtained in three of the populations for the combination *rpo*CC-*Hin*fI are shown in Fig. 1. Population KE (Germany) has six restriction patterns (B, C, D, E, F, G) of which three (C, E, F) are unique to it. All individuals of GA (Scotland) and HH (Sweden) have the same restriction pattern with *rpo*CC-*Hin*fI (Fig. 1). Of the 92 scorable fragments, 44 (48%) are polymorphic bands. The range of size of polymorphic fragments analyzed in different primer-restriction enzyme combinations varied between 190 bp and 950 bp. In all, 24 cpDNA haplotypes were found (data can be obtained from the authors) in the seven populations consisting of 62 individuals. Therefore, 38.7% of the total individuals represent the 24 haplotypes.

Six of the seven populations are polymorphic and all of the six have unique haplotypes, in addition to common haplotypes. The population GA (Scotland) is monomorphic, represented by only one haplotype (H2). The maximum number of haplotypes (eight) as well as that of unique haplotypes (seven) are in the KE (Germany) population. H2 is the most common haplotype, and is present in five of the seven populations. Also, the frequency of this haplotype is highest (0.408), followed by H1 (0.186). The frequency of the remainder of the haplotypes is less than 0.05 (Table 3). There is no haplotype which is shared by all seven populations. Four (H1, H2, H4, H7) of 24 haplotypes are shared by two or more populations (Table 3). MS (Spain) is the only population that contains these four haplotypes, in addition to other unique haplotypes.

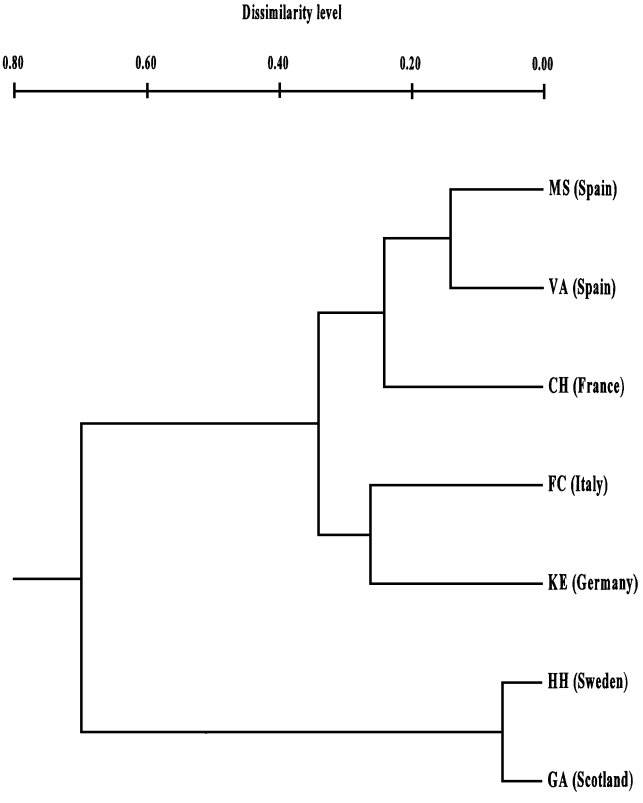
The dendrogram obtained by the UPGMA method using the frequencies of haplotypes in each population demonstrates the relatedness amongst the populations and their geographical locations (Fig. 2). Two major clusters separated by a dissimilarity level of 70% was observed; the northern Europe populations (HH and GA) belonged to one cluster and the rest of populations to the other. The two populations from Spain (MS and VA) are grouped together and population CH (France) joins them. FC (Italy) and KE (Germany) form another sub-

Table 3 Haplotype frequencies and composition of the seven populations of *P. spinosa*

Haplotypes	Populations[a]							Individuals/ haplotype	Frequency[b]
	MS	VA	CH	FC	KE	HH	GA		
H1	3	2	0	4	2	0	0	11	0.186
H2	2	2	5	0	0	8	6	23	0.408
H3	1	0	0	0	0	0	0	1	0.014
H4	1	0	1	0	0	0	0	2	0.027
H5	1	0	0	0	0	0	0	1	0.014
H6	1	0	0	0	0	0	0	1	0.014
H7	1	0	2	0	0	0	0	3	0.040
H8	0	0	1	0	0	0	0	1	0.013
H9	0	0	1	0	0	0	0	1	0.013
H10	0	0	1	0	0	0	0	1	0.013
H11	0	0	0	3	0	0	0	3	0.043
H12	0	0	0	1	0	0	0	1	0.014
H13	0	0	0	1	0	0	0	1	0.014
H14	0	0	0	1	0	0	0	1	0.014
H15	0	0	0	0	1	0	0	1	0.014
H16	0	0	0	0	1	0	0	1	0.014
H17	0	0	0	0	2	0	0	2	0.028
H18	0	0	0	0	1	0	0	1	0.014
H19	0	0	0	0	1	0	0	1	0.014
H20	0	0	0	0	1	0	0	1	0.014
H21	0	0	0	0	1	0	0	1	0.014
H22	0	0	0	0	0	1	0	1	0.014
H23	0	0	0	0	0	1	0	1	0.014
H24	0	1	0	0	0	0	0	1	0.028
Totals	10	5	11	10	10	10	6	62	1.000

[a] MS=Montejo de la Sierra; VA=Valdemorillo; CH=Chizé; FC=Parco Nazionale delle Foreste Casentinesi; KE=Kelheim; HH=Halltorps Hage; GA=Glen Affric

[b] Calculated by the HAPLODIV program (Pons and Petit 1995)



group and are close (dissimilarity only 34%) to the latter three populations, MS, VA and CH (Fig. 2).

Results of the analysis of cpDNA diversity using the HAPLODIV and HAPLONST programs are shown in Table 4. The total diversity (h_r) is high (0.824). The major portion of this total diversity is within the populations (h_s = 0.663). The level of population subdivision of diversity using unordered alleles is low (G_{ST} = 0.195) and using ordered alleles is still lower (N_{ST} = 0.136). The minimum-length spanning-tree of haplotypes does not show any correlation between the phylogeny of haplotypes and their geographic locations (Fig. 3). However, two main clusters of haplotypes could be observed with H2 and H10 as the internal nodes. H2 (the most abundant haplotype) is related to H10 by a single mutation (K1K2-*Hin*fI = III; Fig. 3). The geographically related as well as the unrelated haplotypes belong to both the clusters of H2 and H10, thus exhibiting a random phylogenetic distribution of the haplotypes.

Fig. 2 Dendrogram of seven populations of *P. spinosa* generated by UPGMA clustering analysis using the "Euclidean distances squared" coefficient. *MS* Montejo de la Sierra; *VA* = Valdemorillo; *CH* = Chizé; *FC* = Parco Nazionale delle Foreste Casentinesi; *KE* = Kelheim; *HH* = Halltorps Hage; *GA* = Glen Affric

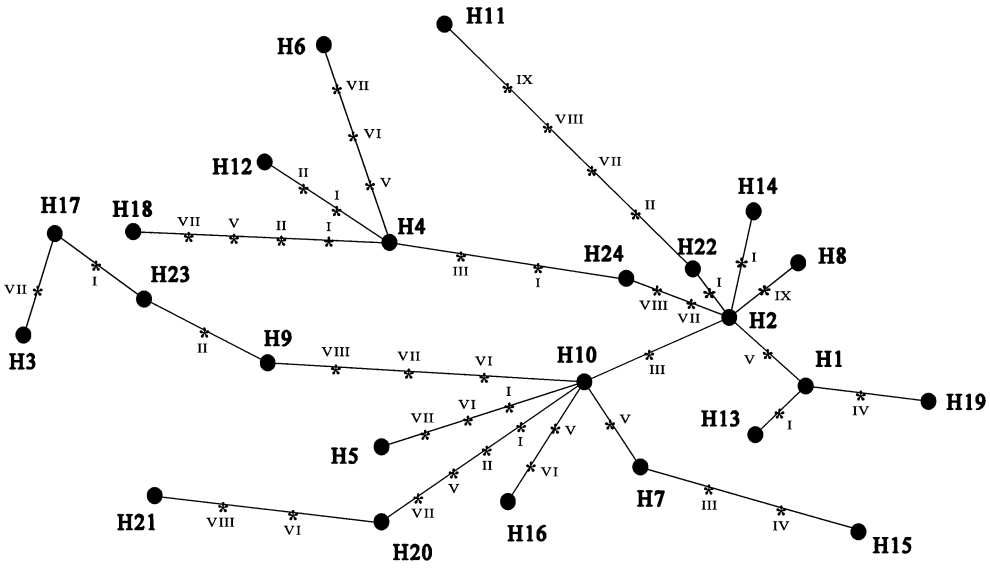


Fig. 3 Minimum-length spanning tree of 24 cpDNA haplotypes of *P. spinosa* from seven European deciduous forests. The *asterisks with Roman numerals* represent restriction pattern differences between haplotypes for each primer-restriction enzyme combination. *I* = HK-*Alu*I, *II* = HK-*Hin*fI, *III* = K1K2-*Hin*fI, *IV* = K1K2-*Taq*I, *V* = *rpo*CC-*Hin*fI, *VI* = CD-*Alu*I, *VII* = CD-*Hin*fI, *VIII* = CD-*Taq*I, *IX* = VL-*Alu*I

Table 4 Results of the analysis of diversity; standard errors of the estimates are in parentheses

h_S	0.663 (0.131)
h_T	0.824 (0.096)
G_{ST}	**0.195 (0.104)**
v_S	0.533 (0.147)
v_T	0.617 (0.153)
N_{ST}	**0.136 (NC)**[a]

[a] Not computed

Discussion

In the present investigation, high total cpDNA diversity (h_T = 0.824) was detected in *P. spinosa*; 44 mutations are distributed over 24 haplotypes. *P. spinosa* is a wild shrub which has not undergone any selection pressure imposed by man for its domestication. In the absence of such selection pressure, it is most probable that cytoplasmic diversity (in the present study, the cpDNA variability) is maintained in nature. Also, high cpDNA variation maybe related to a high generation turnover of this shrub species and hence a greater probability for mutations to be fixed in later generations.

The origin of the cytoplasm of the allotetraploid *P. spinosa* is not known. It has been shown that *Prunus cerasifera* is one of the parents of *P. spinosa* (Reynders-Aloisi and Gellet 1994) but the second parent is uncertain. Moreover, *P. cerasifera* itself has diploid, tetraploid and hexaploid forms (Beridze and Kvatchadze 1981; Watkins 1981). It is not yet known whether *P. cerasifera* has acted as pollen donor or cytoplasm donor in the evolution of *P. spinosa.* It is also possible that both *P. cerasifera* (diploid or/and polyploid forms) and the second unknown parent may have acted as male as well as female parent at different times in the evolution of *P. spinosa.* Such an evolutionary history of *P. spinosa* maybe one of the reasons for its raised cpDNA diversity.

The intrapopulation variations are higher in southern Europe compared to northern Europe populations. Population GA (Scotland) is homogeneous with no intrapopulation variation, while HH had three haplotypes (H2, H22 and H23) of which two (H22 and H23) are unique to this population. The per-cent individuals showing distinct haplotypes per population of the studied southern european deciduous forests range between 50% (i.e. five haplotypes in ten individuals in Italy) and 80% (i.e. eight haplotypes in ten individuals in Germany). This suggests that *P. spinosa* occupied southern Europe earlier compared to northern parts. Such a higher diversity of haplotypes in southern Europe is in agreement with previous reports in *Fagus* (Demesure et al. 1996). *Quercus* (Dumolin et al. 1997a) and *Alnus* (King and Ferris 1998).

The six polymorphic populations, in addition to the common haplotypes (i.e. H1, H2, H4 and/or H7), have their own unique or ”private” haplotypes as denominated by Slatkin (1985). These population-specific haplotypes can serve to distinguish different populations. The low frequency (less than 0.05) of these haplotypes suggests that they may be the result of recent mutations. On the other hand, the high frequency and wide geographical distribution of haplotype H2, probably reflects its ancient origin.

The dendrogram showing the closeness between the populations could be correlated to their geographical positions. The northern Europe populations GA (Scotland) and HH (Sweden), which are comparatively more homogeneous in their haplotype constitution, are more similar to each other but are most distant to the rest of the populations FC (Italy) and KE (Germany) which have the higher number of ”private” haplotypes, form one subgroup and join the other sub-group with populations from Spain (MS and VA) and France (CH).

The major portion of the total diversity (h_T = 0.824) is located within populations (h_S = 0.663). The G_{ST} value is low (19.5%), compared to other forest species such as *Argania spinosa* (60%, El Mousadik and Petit 1996), *Fagus sylvatica* (83%, Demesure et al. 1996) and *Alnus glutinosa* (86%, King and Ferris 1998). However, very low genetic differentiation among populations (5%) has

been observed in the highly polymorphic *Trifolium pratense* (Milligan 1991). According to Pons and Petit (1996), G_{ST} values maybe biased at very polymorphic loci, which may also be the case for the present results; the N_{ST} value (an analogue of G_{ST}) is more useful in such cases. A low N_{ST} value (13.6%) was observed in the present study demonstrating that differentiation among populations is actually low. N_{ST} lower than G_{ST} indicates an absence of phylogeographic structure (Pons and Petit 1996). This was indeed the case and no congruency between the phylogeny of haplotypes and their geographical positions could be demonstrated in terms of the studied geographical scale. Incongruency between gene trees and their geographical distribution have been reported earlier in *Eucalyptus nitens* (Byrne and Moran 1994). One of the reasons for this geographical discordance in *P. spinosa* could be chloroplast gene flow among populations. It appears that seed dispersal, especially by birds, could be one of the causes of such gene flow (in the absence of studies on plastid inheritance in *P. spinosa* we assume maternal inheritance of cpDNA, as in most angiosperms, and thus the gene flow of cpDNA would be limited to seed dispersal). At this stage we reserve further comments on the phylogeography of *P. spinosa*. Presently we are planning an extension of this work with more populations of European deciduous forests to reach a more-decisive conclusion on this aspect.

High intraspecific cpDNA polymorphism in *P. spinosa* calls for a note of caution for its use in phylogenetic studies. This feature, however, may not be generalized to other species of this genus (limited polymorphism of cpDNA was observed in wild populations of *Prunus avium*, unpublished results). The conservation strategies for this species in the forests studied have to take into account the presence of ”private” haplotypes in most populations, indicating the uniqueness of these populations.

Acknowledgements We are grateful to Dr. Remy J. petit for providing us the universal primers, statistical programs and for giving helpful suggestions. The research was supported by the EC research program FAIR5-CT97-3795. The experiments comply with the current laws of the country in which the experiments were performed.

References

Banks JA, Birky CW (1985) Chloroplast DNA diversity is low in a wild plant, *Lupinus texensis*. Proc Natl Acad Sci USA 82:6950–6954

Bassam BJ, Caetano-Anollés G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 80:81–84

Beridze RK, Kvatchadze MV (1981) Origin and evolution of cultivated plums of Georgia. Kulturpflanze 29:147–150

Byrne M, Moran GF (1994) Population divergence in the chloroplast genome of *Eucalyptus nitens*. Heredity 73:18–28

Clegg MT, Rawson JR, Thomas K (1984) Chloroplast DNA variation in pearl millet and related species. Genetics 106:449–461

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

Demesure B, Comps B, Petit RJ (1996) Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in europe. Evolution 50:2515–2520

Dumolin-Lapegue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997a) Phylogeographic structure of white oaks throughout the European continent. Genetics 146:1475–1487

Dumolin-Lapegue S, Pemonge M-H, Petit RJ (1997b) An enlarged set of consensus primers for the study of organelle DNA in plants. Mol Ecol 6:393–397

El Mousadik A, Petit RJ (1996) Chloroplast DNA phylogeography of the argan tree of Morocco. Mol Ecol 5:547–555

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species molecular variance parsimony. Genetics 136:343–359

Fernàndez Garcia T, Martín ME, Casp A (1998) Quantification of significant volatile components of Pacharan. Z Lebensm Unters Forsch A 206:414–416

Gaut BS, Muse SV, Clegg MT (1993) Relative rates of nucleotide substitution in the chloroplast genome. Mol Phylog Evol 2:89–96

Gepts P, Clegg MT (1989) Genetic diversity in pearl millet [*Pennisetum glaucum* (L.) R.Br.] at the DNA sequence level: consequences for genetic conservation. J Hered 80:203–208

Gielly L, Taberlet P (1994) The use of chloroplast DNA to resolve plant phylogenies: non-coding vs RbcL sequences. Mol Biol Evol 11:769–777

Guitian J, Guitian P, Sanchez JM (1993) Reproductive biology of *Prunus* species (Rosaceae) in the Northwest Iberian Peninsula. Plant Syst Evol 185:153–165

Jackson HD, Steane DA, Potts BM (1999) Chloroplast DNA evidence for reticulate evolution in *Eucalyptus* (Myrtaceae). Mol Ecol 8:739–751

Kaneko T, Terachi T, Tsunewaki K (1986) Studies on the origin of crop species by restriction endonuclease analysis of organellar DNA. II Restriction analysis of ctDNA of 11 *Prunus species*. Jpn J Genet 61:157–168

King RA, Ferris C (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. Mol Ecol 7:1151–1161

Levy F, Antonovics J, Boyton JE, Gilham NW (1996) A population genetic analysis of chloroplast DNA in *Phacelia*. Heredity 76:143–155

Milligan BG (1991) Chloroplast DNA diversity within and among populations of *Trifolium pratense*. Curr Genet 19:411–416

Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:S6–S29

Petit RJ, Kremer A, Wagner DB (1993) Geographic structure of chloroplast DNA polymorphisms in European oaks. Theor Appl Genet 87:122–128

Pons O, Petit RJ (1995) Estimation, variance and optimal sampling of gene diversity 1. Haploid locus. Theor Appl Genet 90:462–470

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245

Prim RC (1957) Shortest connection networks and some generalizations. Bell Labs Tech J 36:1389–1401

Reynders-Aloisi S, Grellet F (1994) Characterization the ribosomal DNA units in two related *Prunus* species (*P. cerasifera* and *P. spinosa*). Plant Cell Rep 13:641–646

Rohlf FJ (1992) NTSYS-PC: Numerical taxonomy and multivariate analysis system. Exeter Software, New York

Segraves KA, Thompson JN, Soltis PS, Soltis DE (1999) Multiple origins of polyploidy and the geographic structure of *Heuchera grossulariifolia*. Mol Ecol 8:253–262

Slatkin M (1985) Rara alleles as indicators of gene flow. Evolution 39:53–65

Soltis DE, Soltis PS, Milligan BG (1992) Intraspecific chloroplast DNA variation: systematics and phylogenetic implications. In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 117–150

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Terauchi R, Terachi T, Tsunewaki K (1991) Intraspecific variation of chloroplast DNA in *Dioscorea bulbifera* L. Theor Appl Genet 81:461–470

Torres AM, Weeden NF, Martín A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor Appl Genet 85:937–945

Uematsu C, Saskuma T, Ogihara Y (1991) Phylogenetic relationships in the stone fruit group of *Prunus* as revealed by restriction fragment analysis of chloroplast DNA. Jpn J Genet 66:59–66

Van Dijk P, Bakx-Schotman T (1997) Chloroplast DNA phylogeography and cytotype geography in autopolyploid *Plantago media*. Mol Ecol 6:345–352

Watkins R (1976) Cherry, plum, peach, apricot and almond. In: Simmonds NW (ed) Evolution of crop plants. Longman, London, pp 242–247

Watkins R (1981) Plums, apricots, almonds, peaches, cherries (genus *Prunus*). In: Hora B (ed) The Oxford encyclopaedia of trees of the world. Oxford University Press, UK, pp 196–201

Wolfe KH, Wen-Hsiung L, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proc Natl Acad Sci USA 84: 9054–9058

Yeboah GK, Woodell SRJ (1987) Flowering phenology, flower colour and mode of reproduction of *Prunus spinosa* L. (Blackthorn); *Crataegus monogyna* JACQ. (Hawthorn); *Rosa canina* L. (Dog Rose); and *Rubus fruticosus* L. (Bramble) in Oxfordshire. Funct Ecol 1:261–268