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POPULATION GENETIC ANALYSIS OF EUROPEAN *PRUNUS SPINOSA* (ROSACEAE) USING CHLOROPLAST DNA MARKERS[1]

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Chloroplast DNA diversity in *Prunus spinosa*, a common shrub of European deciduous forests, was assessed using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. Thirty-two haplotypes were detected in 25 populations spread across the European continent. Ten haplotypes were shared by two or more populations, and 22 were private. The major proportion of the total cpDNA diversity ($H_T = 0.73$) was located within the populations ($H_S = 0.49$), and differentiation between populations was low ($G_{ST} = 0.33$) compared with other forest species. Haplotype diversity was higher in southern Europe than in northern Europe, indicating probable localization of glacial refugia in southern Europe. The minimum-length spanning tree of haplotypes showed incongruency between the phylogeny of haplotypes and their geographic locations. This might be the result of intensive seed movements following recolonization, which thereby erased the phylogeographic structure in *P. spinosa*.

Key words: CAPS; cpDNA diversity; Europe; PCR-RFLP; phylogeography; private haplotypes; *Prunus spinosa*; Rosaceae.

The chloroplast genome has been considered a conservative molecule (Wolfe, Hsiung, and Sharp, 1987), and hence the probability of detecting intraspecific variation is low. This limitation has now been largely overcome by use of the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique (or CAPS, cleaved amplified polymorphic sequence), which employs universal primers (Taberlet et al., 1991; Demesure, Sodzi, and Petit, 1995; Dumolin-Lapegue, Pemonge, and Petit, 1997) for amplification of specific noncoding sequences of chloroplast DNA, followed by their restriction digestion. This technique reveals a larger number of variations compared with traditional RFLP, since at least 50% of cpDNA variations are due to small insertions and deletions (Gielly and Taberlet, 1994). Intraspecific cpDNA variation, revealed by PCR-RFLP, is increasingly being used for population genetic and phylogeographic studies in plants (Demesure, Comps, and Petit, 1996; El Mousadik and Petit, 1996; King and Ferris, 1998; Newton et al., 1999; Dutech, Maggia, and Joly, 2000; Fineschi et al., 2000; Mohanty, Martín, and Aguinagalde, 2001).

Prunus spinosa L. is an allotetraploid (Reynders-Aloisi and Grellet, 1994) wild shrub commonly found in European deciduous forests. The species is insect-pollinated, and seed dispersal is by mammals and birds (Yeboah and Woodell, 1987; Guitian, Guitian, and Sánchez, 1993). It is resistant to cold, drought, and calcareous soils. These traits are useful for improvement of rootstocks or varieties of plums through interspecific hybridization, as *P. spinosa* represents one of the ancestors of *P. domestica* L. (Watkins, 1976, 1981). The fruits are used for preparation of alcoholic drinks known as ''pacharan'' in Spain (Fernández-García, Martín, and Casp, 1998). The medicinal properties of leaf and fruit extracts render them suitable for preparation of ayurvedic medicines.

The only previous study of genetic variation in this species assessed cpDNA diversity in seven populations occurring in European deciduous forests (Mohanty, Martín, and Aguinagalde, 2000). The present investigation is the first to present a complete picture of cpDNA structuring in 25 wild populations of *P. spinosa* collected across a wide portion of Europe. Our main objectives were to determine whether a phylogeographic structure exists in *P. spinosa* and to analyze the phylogenetic relationships among the cpDNA haplotypes.

MATERIALS AND METHODS

Plant material—Twenty-five wild populations of *P. spinosa* were sampled from deciduous forests across Europe (Table 1). Plants in a population were collected from within a range of 50 km, and the distance between sampled individuals in each population was at least 200 m. Fresh leaves were collected from plants in the field, then frozen and stored at −80°C. All the populations were analyzed with the PCR-RFLP technique, whereas only six populations (populations 1, 6, 9, 11, 17, 18) were screened with conserved chloroplast microsatellite primers (ccmps).

DNA extraction, amplification, and digestion—DNA was extracted from frozen leaves following the protocol of Torres, Weeden, and Martín (1993) and then standardized (4 ng/μL). For amplification of chloroplast microsatellite regions, six pairs of conserved chloroplast microsatellite primers (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7, and ccmp10, as described in Weising and Gardner, 1999) were used. Reactions were carried out in 25 μL of a solution consisting of 10 ng of template DNA, 2 mmol/L of $MgCl_2$, 100 μmol/L of dNTP, 0.2 μmol/L of each primer, and 1 unit of *Taq* polymerase with its respective buffer (*Taq* polymerase and 10× buffer were purchased from Pharmacia Biotech., Brussels, Belgium). Polymerase chain reaction was performed using a Perkin Elmer 9600 thermal cycler (Perkin-Elmer, Norwalk, Connecticut, USA). An initial 5-min denaturation at 94°C was followed by 30 cycles of 94°C for 1 min and 30 s of annealing at 50°C and extension at 72°C for 1 min. Amplification cycles were followed by a final 7-min extension at 72°C. Amplification products of chloroplast microsatellites regions were

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TABLE 1. Location and size of the *Prunus spinosa* study populations.

Population (code and location)	Geographic coordinates	No. of individuals
1. Glen Afric, Great Britain	04°83′ W, 57°32′ N	6
2. Lake District, Great Britain	03°00′ W, 54°27′ N	9
3. Forest of Dean, Great Britain	02°65′ W, 51°83′ N	9
4. Tofta, Sweden	11°70′ E, 57°87′ N	3
5. Stenshuvud, Sweden	14°25′ E, 55°65′ N	6
6. Halltorps Hage, Sweden	16°53′ E, 56°75′ N	10
7. Shönberg, Germany	07°83′ E, 47°96′ N	6
8. Bovenden, Germany	10°05′ E, 51°57′ N	7
9. Kelheim, Germany	11°83′ E, 48°93′ N	10
10. Fointainebleau, France	02°67′ E, 48°42′ N	7
11. Chizé, France	00°40′ W, 46°14′ N	11
12. Seillon, France	05°00′ E, 46°00′ N	8
13. Valbonne, France	04°55′ E, 44°24′ N	10
14. State Forest of Aitone, France	08°88′ E, 42°28′ N	9
15. Devesa da Rogueira, Spain	07°08′ W, 42°25′ N	10
16. Valle de Salazar, Spain	00°92′ W, 42°83′ N	9
17. Montejo de la Sierra, Spain	03°50′ W, 41°13′ N	10
18. Parco Nazionale Foreste Casentinesi, Italy	11°80′ E, 43°78′ N	10
19. Alto Garda Bresciano, Italy	10°88′ E, 45°80′ N	8
20. Park of Calabria, Italy	16°58′ E, 39°00′ N	9
21. Mt. Medvenica, Croatia	15°95′ E, 45°87′ N	7
22. Savarsin, Romania	22°23′ E, 46°02′ N	5
23. Boki, Slovakia	19°12′ E, 48°57′ N	5
24. Paleochori, Greece	23°69′ E, 40°51′ N	8
25. Voronez Reserve, Russia	39°50′ E, 51°83′ N	11

fractionated and visualized using an ALF automatic sequencer (Pharmacia Biotech.), as described by Vendramin et al. (1996).

The details of amplification and restriction digestion conditions for the PCR-RFLP technique are described by Mohanty, Martín, and Aguinagalde (2000). A preliminary study of *P. spinosa* with five primer pairs indicated high cpDNA diversity (Mohanty, Martín, and Aguinagalde, 2000). On the basis of this previous study, selection of three cpDNA primer pairs (HK, K1K2, and VL, as described in Dumolin-Lapegue, Pemonge, and Petit, 1997) for the present study was sufficient to detect as many as 17 polymorphic fragments (Table 2). The amplified products were digested with the restriction enzymes *Hin*fI and *Taq*I (Amersham, Buckinghamshire, UK). In addition, *Alu*I was used with the primer pair HK and VL. 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 under UV light. The size of the polymorphic bands was analyzed using Kodak Digital Science 1D Image Analysis software (Kodak, Rochester, New York, USA), and a 50-base pair (bp) ladder (Pharmacia Biotech.) was used as a molecular size marker.

Analysis of data—The program HAPLODIV (Pons and Petit, 1995) was used to calculate the frequency of the haplotypes and estimate parameters of cpDNA diversity (H_T = total diversity, H_S = average intrapopulation diversity, and G_{ST} = level of population subdivision using unordered alleles) and their standard errors. The program HAPLONST (Pons and Petit, 1996) was used to calculate N_{ST} (level of population subdivision using ordered alleles).

The number of mutational differences between haplotypes of wild populations was calculated to produce a minimum-length spanning tree of haplotypes, using the program NTSYS-pc (Rohlf, 1992). The procedure is used to connect points (haplotypes) by direct links that have the shortest possible total length (Prim, 1957). Minimum spanning networks are alternatives to Wagner

TABLE 2. Major patterns and variants in the polymorphic fragments obtained with different primer-restriction enzyme combinations in *Prunus spinosa*.

Polymorphic fragments	Major pattern[a] → Variant[a] (bp)
HK-(*Hin*fI)1	700(A) → 660(B), 640(C)
HK-(*Hin*fI)2	240(C) → 260(A), 250(B)
HK-*Alu*I	380(A) → 370(B), 350(C), 340(D)
K1K2-(*Hin*fI)1	490(A) → 360(B), 260 + 230(C)
K1K2-(*Hin*fI)2	380(B) → 450(A), 370(C)
K1K2-(*Hin*fI)3	340(C) → 365(A), 350(B)
K1K2-(*Hin*fI)4	190(C) → 205(A), 195(B)
K1K2-(*Taq*I)1	650(D) → 700(A), 670(B), 660(C)
K1K2-(*Taq*I)2	240 + 130(D) → 380 + 370(A), 370 + 350(B), 340 + 240 + 130(C)
K1K2-(*Taq*I)3	290(A) → 280(B), 190 + 150(C), 170 + 130(D), 0(E)
K1K2-(*Taq*I)4	260(A) → 250(B), 180(C)
VL-(*Hin*fI)1	440(B) → 460(A)
VL-(*Hin*fI)2	370(A) → 340(B)
VL-*Taq*I	1000(A) → 500 + 500(B)
VL-(*Alu*I)1	900(A) → 880(B)
VL-(*Alu*I)2	670(A) → 400 + 270(B)
VL-(*Alu*I)3	560(B) → 570(A), 530(C), 380 + 180(D)

[a] A, B, C, D, and E are the polymorphisms in each polymorphic fragment.

TABLE 3. Composition of the haplotypes obtained from analysis of six chloroplast microsatellites in *Prunus spinosa*; ccmp3 produced polymorphic fragments, while ccmp2, ccmp4, ccmp6, ccmp7, and ccmp10 produced monomorphic fragments.

Haplotype	Fragments (in bp) produced with conserved chloroplast microsatellite primers					
	ccmp2	ccmp3	ccmp4	ccmp6	ccmp7	ccmp10
I	200	111	120	114	134	114
II	200	112	120	114	134	114
III	200	113	120	114	134	114

parsimony trees and better convey the connections between haplotypes (Excoffier and Smouse, 1994).

RESULTS

Six populations were screened for polmorphisms in chloroplast microsatellite regions, using six pairs of conserved chloroplast microsatellite primers (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7, and ccmp10). The length of one (ccmp3) of the six primer pairs varied by up to 2 bp. In all, three haplotypes were found (Table 3). Haplotype I was the most frequent and abundant in all the populations studied. Haplotype II was detected in populations 9 (two individuals), 11 (four individuals), and 17 (one individual), and haplotype III in populations 9 (one individual) and 17 (one individual).

For the complete survey of 25 populations, three pairs of universal primers (HK, K1K2, and VL) were used to amplify three regions of cpDNA. The sizes of the fragments amplified by HK, K1K2, and VL were 1700 bp, 2650 bp, and 3900 bp, respectively (Mohanty, Martín, and Aguinagalde, 2000), or a total of 8250 bp. Restriction digestion of these fragments produced 17 scorable polymorphic fragments, revealing mainly length variations and few point mutations (Table 2). Figure 1 shows some of the restriction patterns observed in the combination K1K2-*Hin*fI. The combination of all the mutations resulted in 32 haplotypes in the 25 populations, which consisted of 203 individuals (Table 4). Of the 32 haplotypes, ten are shared by two or more populations, and 22 were private (Table 4). Haplotype H19 was the most frequent (0.493) and widely distributed (present in 20 of 25 populations), followed by H10 (frequency = 0.113) which was present in 10 of the 25 populations. The frequency of the private haplotypes varied between 0.005 and 0.044 (Table 4). The private haplotypes H31 and H32 were abundant in populations 25 (Russia) and 20 (Italy), respectively. Population 9 (Germany) had the maximum number (seven) of haplotypes. The maximum number of private haplotypes (three) was present in populations 9 (Germany), 14 (France), and 25 (Russia).

Nineteen of the 25 populations were polymorphic. Among the six monomorphic populations, five were represented by haplotype H19 (the most frequent and abundant haplotype). Of these five monomorphic populations, three (1, 4, 5) were from northern Europe and two (8, 13) from southern Europe. Population 20 (Italy) was the only monomorphic population represented by a private haplotype, H32.

The results of analysis of diversity are presented in Table 5. Total diversity was high ($H_T = 0.73$), the major portion of which was due to intrapopulation diversity ($H_S = 0.49$). The level of population subdivision using unordered and ordered alleles was $G_{ST} = 0.33$ and $N_{ST} = 0.49$, respectively. The difference between N_{ST} and G_{ST} was nonsignificant (U test = 0.98, $P = 0.05$; Pons and Petit, 1996).

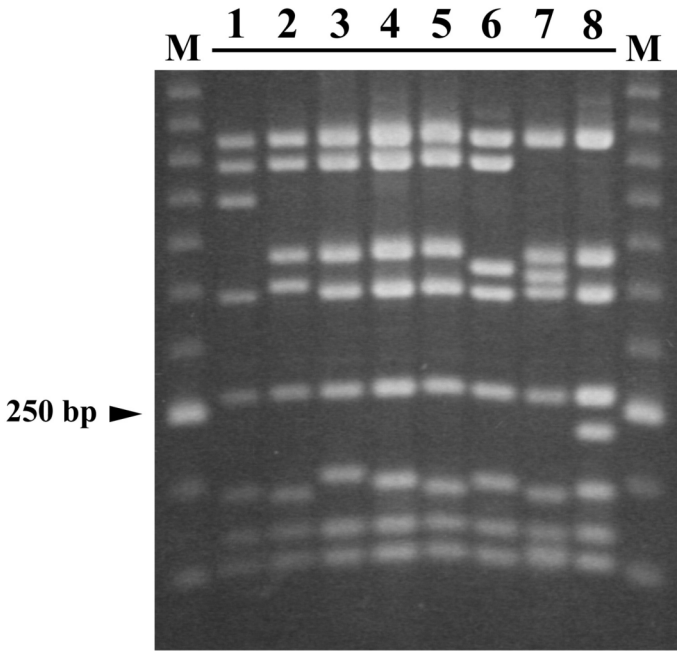

Fig. 1. Eight restriction patterns detected with the primer-enzyme combination K1K2-*Hin*fI in *Prunus spinosa*, resolved on agarose gel. M = molecular size marker (50 base pair ladder, Pharmacia Biotech.).

The phylogenetic relationships between the 32 haplotypes is depicted in the minimum-length spanning tree in Fig. 2. Haplotypes H10, H13, and H19 form three nodes of the tree. The most frequent and abundant haplotype, H19, differs from H10 by one mutation, and H10 differs from H13 by three mutations. The tree does not show a correlation between the phylogeny of the haplotypes and their geographic locations.

DISCUSSION

Our analysis of cpDNA microsatellites using six conserved chloroplast microsatellite primers revealed three haplotypes in 57 individuals belonging to six populations (1, 6, 9, 11, 17, 18). In contrast, a previous study (Mohanty, Martín, and Aguinagalde, 2000) that used the PCR-RFLP technique, in which the noncoding regions of cpDNA are amplified followed by restriction digestion, detected 24 haplotypes in seven populations (62 individuals), six (1, 6, 9, 11, 17, 18) of which were common to the present study. Owing to the higher efficiency of the PCR-RFLP technique in detecting cpDNA variation in *P. spinosa* compared with chloroplast microsatellite regions (using ccmps), the complete investigation was carried out by the former method. The difficulty in detecting intraspecific polymorphisms in microsatellite regions of cpDNA has been discussed by Weising and Gardner (1999) and Provan, Powell, and Hollingsworth (2001).

The survey of 25 populations of *P. spinosa*, using the PCR-RFLP technique, revealed high cpDNA diversity ($H_T = 0.73$). The analysis of only 6.9% of the chloroplast genome (considering cpDNA size in *Prunus* sp. is ~140 kbp; Kaneko, Terachi, and Tsunewaki, 1986) revealed 32 haplotypes. Twenty-four haplotypes were found when 12% of cpDNA was analyzed in seven populations of this species (Mohanty, Martín, and Aguinagalde, 2000). Of the 32 haplotypes, one (H19) was the most frequent (0.493) and abundant in 20 of 25 popula-

TABLE 4. Haplotype frequencies and composition of the 25 populations of *Prunus spinosa*.

Haplotype	Population (no. of individuals with haplotype)																									Total no. of individuals with haplotype	Frequency
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
H1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.005
H2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	3	0.015
H6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0.005
H8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0.010
H9	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	4	0.020
H10	0	2	2	0	0	0	0	0	0	1	3	0	0	0	2	3	1	0	2	0	0	0	1	6	0	23	0.113
H11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0.005
H12	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	3	0	0	0	0	1	0	0	0	5	0.025
H13	0	0	0	0	0	1	0	0	2	0	0	0	0	0	7	1	1	0	0	0	0	0	0	0	1	13	0.064
H14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.005
H15	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3	0.015
H17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	0.015
H18	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H19	6	5	7	3	6	9	1	7	2	5	6	7	10	5	0	2	5	5	6	0	2	1	0	0	0	100	0.493
H20	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	0.010
H21	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3	0.015
H22	0	0	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.020
H23	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2	0.010
H25	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.010
H26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	3	0.015
H27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0.005
H28	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3	0.015
H29	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0.005
H30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0.005
H31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5	0.025
H32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	9	0.044
Totals	6	9	9	3	6	10	6	7	10	7	11	8	10	9	10	9	10	10	8	9	7	5	5	8	11	203	1.000

Note: Locations of populations, by number, are given in Table 1.

TABLE 5. Results of the analysis of diversity in populations of *Prunus spinosa*. Standard errors of the estimates are in parentheses.

Measure	Result
No. of populations	25
Arithmetic mean	8.04
Harmonic mean	7.35
H_S	0.49 (0.07)
H_T	0.73 (0.07)
G_{ST}	0.33 (0.07)
v_S	0.38 (0.06)
v_T	0.73 (0.18)
N_{ST}	0.48 (0.14)

tions. It also formed an internal node in the minimum-length spanning tree, probably reflecting its ancient origin. In general, the private haplotypes were present in low numbers in the study populations; however, H31 and H32 were abundant in populations 25 and 20, respectively, and H19 was lacking. These two populations can be characterized by the dominance of these unique haplotypes.

The populations of northern Europe (Great Britain, Sweden, and Russia: populations 1–6, 25) had 7 haplotypes, of which 4 were private, whereas the populations of southern Europe (the remaining populations) had 29 haplotypes, 18 of which were private. Furthermore, of the seven populations of northern Europe, three (1, 4, 5) were monomorphic and contained the most frequent haplotype (H19), while only two (8, 13) of the 18 populations of southern Europe were monomorphic with haplotype H19. The other unique monomorphic population of southern Europe was 20 (Italy), which consisted of the private haplotype H32. The decrease in heterogeneity of haplotype diversity from southern Europe to northern Europe matches the findings of several other studies (Demesure, Comps, and Petit, 1996; King and Ferris, 1998; Ferris, King, and Hewitt, 1999). In these studies, the molecular data were supported by evidence from fossil pollen records (Huntley and Birks, 1983; Bennett, Tzedakis, and Willis, 1991) that indicated that species such as *Quercus* sp., *Fagus sylvatica* L., and *Alnus* sp. had refugia in southern Europe during the last glaciation. In the absence of fossil pollen records for *Prunus* species, the high haplotype diversity of *P. spinosa* in southern Europe is the only strong indication of a possible refugium in this region.

The partition of genetic diversity among the *P. spinosa* populations is low ($G_{ST} = 0.33$) compared with the average ($G_{ST} = 0.70$) for 97 plant species (Petit, 1999). However, it is comparable to that for *Prunus avium* L. ($G_{ST} = 0.29$; Mohanty, Martín, and Aguinagalde, 2001). The N_{ST} value was higher (0.48) than the G_{ST} value, but the difference was not significant (U test = 0.98, $P = 0.05$). This finding indicates an incongruency between the phylogeny of haplotypes and their geographic locations (Pons and Petit, 1996). One of the reasons for such incongruency could be intensive seed movements since recolonization, which has erased the geographic structure. We assumed that cytoplasmic gene flow in *P. spinosa* is restricted to seed dispersal, as in most angiosperms (Birky, 1995). The fruits of this shrub are ingested and dispersed by mammals and to some extent by birds (Yeboah and Woodell, 1987; Guitian, Guitian, and Sánchez, 1993). The long-distance seed dispersal in *P. spinosa* has to be efficient enough to decrease the genetic heterogeneity among populations and erase the phylogeographic structure. Several studies have suggested

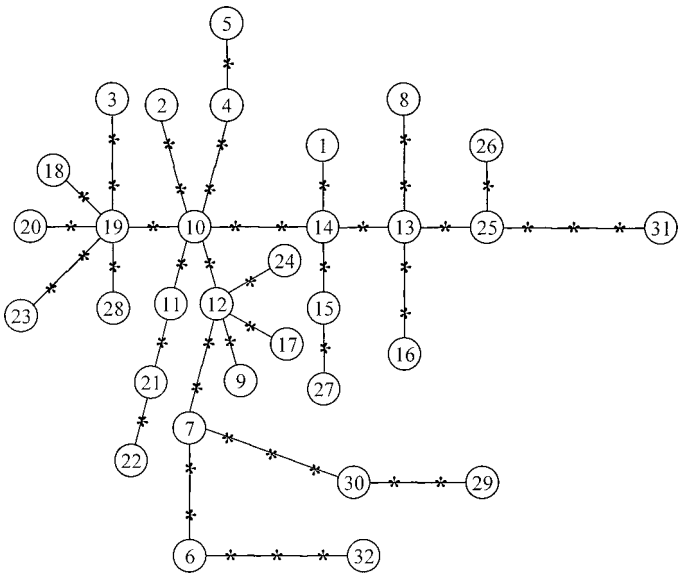

Fig. 2. Minimum-length spanning tree of 32 cpDNA haplotypes of *Prunus spinosa* from 25 European deciduous forests. Numbers 1–32 in the circles correspond to haplotypes H1–H32, respectively. Each asterisk represents one restriction pattern difference between haplotypes.

that long-distance gene flow between plant populations is not as infrequent as once thought (Ellstrand, Devlin, and Marshall, 1989; Dow and Ashley, 1996; Dawson et al., 1997).

The phylogenetic relationships between the haplotypes can be analyzed from the minimum-length spanning tree. A possible inference from the tree is that haplotypes H10, H13, and H19 represent three nodes, for the following reasons: (1) there is no population in which at least one of the three haplotypes does not exist (except population 20, in which all individuals are represented by the private haplotype H32); and (2) when H19 is absent from populations 15 and 24, then the dominating haplotypes are H13 and H10, respectively. In population 25 (in which H10 and H19 are absent), H13 is present although not dominant. Here, the private haplotype H31 is dominant and occupies the tip of the phylogenetic tree. It is possible that H31 has gained selective advantage over H13, which is now scarce in the population. The population of Slovakia (23) lacks H19 and H13 but has H10. Furthermore, the three haplotypes (H19, H13, and H10) have higher frequencies compared to rest of the haplotypes and therefore can be termed as nodes of the minimum-length spanning tree. However, there is a disparity between the frequency of H19 and the frequencies of H10 and H13, indicating that H19 is of ancient origin. This inference is further supported by the fact that H19 is the most abundant and geographically widespread haplotype. The node H10 is linked to H12 by a single mutation. H12 may be called a subnode, as it harbors a separate group of eight haplotypes, none of which are present in the populations of northern Europe.

The present study has contributed to the growing understanding of the phylogeography of temperate plant species in European deciduous forests. In the absence of fossil pollen data for *P. spinosa*, population genetic analysis of the chloroplast genome, the extent of haplotype diversity specifically, is the first attempt to indicate the existence of glacial refugia for the species in southern Europe. The private haplotypes in the populations give them special features, and policy makers

must consider these when formulating guidelines for conservation and management of this species.

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