A population genetic analysis of chloroplast DNA in wild populations of *Prunus avium* L. in Europe

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A population genetic study of chloroplast DNA was carried out in 23 wild populations of *Prunus avium* sampled from several European deciduous forests. An analysis of approx. 9% of the chloroplast genome detected mostly insertion–deletion mutations and one point mutation. In all, 16 haplotypes were detected. Six haplotypes were shared by two or more populations and 10 were unique. One haplotype was present in 21 of the 23 populations and 161 of 211 individuals, which probably indicates its ancient origin. The level of population subdivision, using unordered and ordered alleles, was low, $G_{STC} = 0.29$ and $N_{STC} = 0.33$, respectively. The difference between G_{STC} and N_{STC} is nonsignificant, indicating an absence of correlation between haplotype phylogeny and geographical distribution. The absence of phylogeographic structure in wild cherry may be attributed to long distance gene flow among populations by birds, animals and anthropogenic activities. The minimum-length spanning tree depicting the phylogenetic relationships between the haplotypes indicates the possible existence of two lineages represented by the haplotypes H3 and H4. The information about homogeneity or heterogeneity of populations in terms of haplotype constitution and detection of rare haplotypes in some populations will be useful for formulation of conservation and management strategies of wild cherry.

Keywords: cpDNA diversity, PCR-RFLP, phylogenetic relations, *Prunus avium* L., universal primers, wild cherry.

Introduction

Intraspecific chloroplast DNA (cpDNA) diversity is very useful in determining population structure and in phylogeographic studies (McCauley, 1995; Demesure *et al.*, 1996; El Mousadik & Petit, 1996; King & Ferris, 1998; Dutech *et al.*, 2000; Fineschi *et al.*, 2000). Such studies have great implications for forest conservation and management (Newton *et al.*, 1999). PCR-RFLP (polymerase chain reaction–restriction fragment length polymorphism) using universal primers (Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997) has greatly facilitated the detection of intraspecific cpDNA variation. Fifty percent of such variations are attributable to small insertions and deletions (Gielly & Taberlet, 1994).

Prunus avium L., commonly known as wild cherry, is an important noble hardwood species of European deciduous forests (Turok *et al.*, 1996). It is an important

timber, fruit and rootstock species. Furthermore, it is the wild ancestor of sweet cherry cultivars and has been used in genetic improvement programmes (Tydeman *et al.*, 1966; Tobutt, 1985; Nicoll, 1993). The species is self-incompatible, insect-pollinated and seed dispersal is by animals, birds and humans (Crane & Lawrence, 1931; Crane & Brown, 1937). Nuclear genetic variability studies have been carried out in both cultivated and wild cherry (Frascaria *et al.*, 1993; Beaver *et al.*, 1995; Mariette *et al.*, 1997). Mohanty *et al.* (2001) found low genetic differentiation among five wild cherry populations. However, these studies were limited by the number of populations (five to six) and distributional area investigated. The present investigation is the first attempt to present a more complete picture of cpDNA structuring in wild populations of *P. avium* by including 23 wild populations collected over a wide European range. The main objectives were to determine whether a phylogeographic structure exists in *P. avium* and to infer the phylogenetic relationships amongst the cpDNA haplotypes.

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Materials and methods

Plant material

Twenty-three wild populations of *P. avium* were sampled from deciduous forests across Europe (Table 1). Leaves were taken from mature trees located deep inside the forest, thus ensuring, to a large extent, that the trees had not been planted and were growing wild. The distance between sampled individuals in each population was at least 200 m. Fresh leaves were collected from plants in the field, frozen and stored at -80°C .

DNA extraction, amplification and digestion

DNA was extracted from frozen leaves following the protocol of Torres *et al.* (1993), and then standardized (4 ng/ μL).

The details of amplification and restriction digestion conditions are described in Mohanty *et al.* (2000, 2001). Five cpDNA primer pairs (HK, K1K2, CD, DT and VL; described in Dumolin-Lapegue *et al.*, 1997) were used in the present study. Amplified products of these primers were digested with the restriction enzymes, *HinfI* and *TaqI* (Amersham). In addition, *AluI* was used with the primer pair VL. Restriction fragments were

separated on 2.6% agarose gels in Tris-borate-EDTA buffer (1 $\times$), run at 3 V/cm for 4 h, stained with ethidium bromide and visualized under UV light. Digests were also resolved on 8% polyacrylamide gels run in TBE buffer (1 $\times$) at 350 V for 4–8 h (time depending on the size of fragment to be resolved); these gels were silver stained (Bassam *et al.*, 1991). The size of the polymorphic bands were analysed using Kodak Digital Science 1D Image Analysis Software, and a 50-bp ladder (Pharmacia) was used as a molecular size marker.

Analysis of data

The program HAPLODIV (Pons & 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_{STC} = level of population subdivision using unordered alleles) and their standard errors. The program HAPLONST (Pons & Petit, 1996) was used to calculate N_{STC} (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 NTSYS-pc (Rohlf, 1992). The procedure is used to connect points (haplotypes) by direct links having the

Table 1 Populations, origin and number of individuals studied in *Prunus avium*

Code	Population	Origin	Longitude, Latitude	No. of individuals
01	Glen Afric	Great Britain	04°83'W, 57°32'N	4
02	Lake District	Great Britain	03°00'W, 54°27'N	7
03	Forest of Dean	Great Britain	02°65'W, 51°83'N	10
04	Tofta	Sweden	11°70'E, 57°87'N	8
05	Stenshuvud	Sweden	14°25'E, 55°65'N	8
06	Shönberg	Germany	07°83'E, 47°96'N	10
07	Bovenden	Germany	10°05'E, 51°57'N	11
08	Kelheim	Germany	11°83'E, 48°93'N	10
09	Fontainebleau	France	02°67'E, 48°42'N	9
10	Chizé	France	00°40'W, 46°14'N	10
11	Seillon	France	05°00'E, 46°00'N	10
12	Valbonne	France	04°55'E, 44°24'N	9
13	State Forest of Aitone	France	08°88'E, 42°28'N	10
14	Devesa da Rogueira	Spain	07°08'W, 42°25'N	10
15	Valle de Salazar	Spain	00°92'W, 42°83'N	15
16	Montejo de la Sierra	Spain	03°50'W, 41°13'N	10
17	Parco Naz. Foreste Casentinesi	Italy	11°80'E, 43°78'N	10
18	Alto Garda Bresciano	Italy	10°88'E, 45°80'N	10
19	Park of Calabria	Italy	16°58'E, 39°00'N	10
20	Mt. Medvenica	Croatia	15°95'E, 45°87'N	4
21	Savarsin	Romania	22°23'E, 46°02'N	3
22	Boki	Slovakia	19°12'E, 48°57'N	13
23	Arnea	Greece	23°60'E, 40°46'N	10

smallest possible total length (Prim, 1957). Minimum spanning networks are alternatives to Wagner parsimony trees, but better convey the connections between haplotypes (Excoffier & Smouse, 1994).

Results

Five pairs of universal primers (HK, K1K2, CD, DT and VL) representing 13.2 kb, approx. 9% of the chloroplast genome, were used for the complete survey. The sizes of the amplified products for HK, K1K2, CD, DT and VL were 1700 bp, 2650 bp, 3800 bp, 1150 bp and 3900 bp, respectively (Mohanty *et al.*, 2001). Of the 11 primer-restriction enzyme combinations, 10 showed resolved polymorphic fragments (Table 2). Of a total of 67 resolved fragments, 41 (60.1%) were polymorphic. All mutations were apparently insertion-deletions, except in the combination VL-*TaqI* where a point mutation was found (Table 2). Figure 1 shows representative restriction patterns observed in the combinations K1K2-*HinfI* and K1K2-*TaqI*. In all, 16 haplotypes were distinguished in the 23 populations (211 individuals). Ten haplotypes (H1, H2, H6, H9, H10, H11, H12, H14, H15 and H16) were unique to their respective populations, whereas the remaining six (H3, H4, H5, H7, H8 and H13) were shared by two or

more populations (Table 3). H3 is the most frequent (0.73) and abundant haplotype, being present in 21 of the 23 populations; it is absent in two of the eastern populations (21, Romania and 23, Greece; Table 3). Populations 02, 03 (Great Britain), 09, 10, 13 (France) and 17 (Italy) were monomorphic for haplotype H3. Populations 04, 05 (Sweden), 06, 07 (Germany), 09, 10, 11, 12, 13 (France), 14, 16 (Spain), 18 (Italy), 20 (Croatia), 21 (Romania) and 23 (Greece) contained no unique haplotypes. The maximum number of haplotypes (five) occurred in population 19 (Italy), of which three were unique, followed by population 15 (Spain) with four haplotypes, of which three were unique (Table 3).

A major proportion of the total diversity ($h_T = 0.46$) is contributed by intrapopulation diversity ($h_S = 0.33$; Table 4). The level of population subdivision using unordered and ordered alleles were $G_{STC} = 0.29$ and $N_{STC} = 0.33$, respectively. The difference between N_{STC} and G_{STC} was nonsignificant (U -test = 0.35, $P = 0.05$, according to Pons & Petit, 1996).

The minimum-length spanning tree showing the phylogenetic relationships between the 16 haplotypes is shown in Fig. 2. Haplotypes H3 and H4 form two nodes of the tree and are linked by the haplotypes H5 and H7, each differing from the adjacent haplotypes by a single mutation. The private haplotypes, H2, H6, H11 and H16, are related to node H3, whereas the haplotypes of population 19 (i.e. H12, H13, H14 and H15), two private haplotypes of population 15 (H9 and H10) and H1 of population 01 are linked to node H4. The shared haplotype, H8, is also related to node H4.

Table 2 Majority patterns and variants (in bp) in the polymorphic fragments obtained with different primer-restriction enzyme combinations in *Prunus avium*. A, B and C are the haplotypes in each polymorphic fragment. Combinations of A, B and/or C produce the final haplotypes (H1 to H16, see text)

Polymorphic fragments	Majority → Variant
HK- <i>HinfI</i>	250(A) → 240(B)
K1K2-(<i>HinfI</i>)1	190(B) → 195(A)
K1K2-(<i>HinfI</i>)2	175(B) → 180(A), 170(C)
K1K2-(<i>TaqI</i>)1	340(B) → 350(A)
K1K2-(<i>TaqI</i>)2	270(A) → 265(B)
K1K2-(<i>TaqI</i>)3	195(B) → 205(A), 190(C)
CD-(<i>HinfI</i>)1	0(B) → 530(A)
CD-(<i>HinfI</i>)2	220(A) → 210(B)
CD-(<i>TaqI</i>)1	850(A) → 600(B)
CD-(<i>TaqI</i>)2	330(A) → 315(B)
DT-(<i>HinfI</i>)1	300(B) → 310(A)
DT-(<i>HinfI</i>)2	275(A) → 270(B)
DT-(<i>TaqI</i>)1	550(B) → 555(A)
DT-(<i>TaqI</i>)2	400(B) → 410(A), 395(C)
VL-(<i>AluI</i>)1	1050(A) → 900(B)
VL-(<i>AluI</i>)2	550(B) → 625(A)
VL- <i>HinfI</i>	380(B) → 390(A)
VL- <i>TaqI</i>	1600(B) → 1700(A), 1200 + 400(C)

Fig. 1 Restriction patterns of primer-enzyme combinations K1K2-*HinfI* (1–6) and K1K2-*TaqI* (7–12), resolved on agarose gel. M indicates the molecular size marker (50 bp ladder, Pharmacia). Arrows indicate the polymorphic sites detected.

Table 3 Haplotype frequencies and composition of the 23 populations of *Prunus avium*

Haplotypes	Populations†																							Individuals/ haplotype	Frequency‡
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
H1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.011
H2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.011
H3	2	7	10	7	4	7	9	6	9	10	9	6	10	9	12	8	10	9	2	3	0	12	0	161	0.732
H4	0	0	0	1	2	2	1	2	0	0	1	1	0	0	0	2	0	1	0	0	0	0	1	14	0.064
H5	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	9	14	0.073
H6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.004
H7	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	3	0.014
H8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	2	0.015
H9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0.003
H10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0.003
H11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0.003
H12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	3	0.013
H13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	3	0.033
H14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	0.009
H15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	0.009
H16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.003
Totals	4	7	10	8	8	10	11	10	9	10	10	9	10	10	15	10	10	10	10	4	3	13	10	211	1.000

† Population codes in Table 1.

‡ Calculated by HAPLODIV program (Pons & Petit, 1995).

subnode H4. Haplotype H4 is absent in all these populations.

Haplotype H13 is linked to H4 by four mutations and the rest of the haplotypes of this lineage (except H1 and H8) are relatively distantly related to each other (two to three mutational differences) and to H4. Similarly, the unique haplotypes H11 and H16 are four and five mutations away from the node H3, respectively. The larger number of mutational differences of rare haplotypes from nodes H3 and H4 may be due to missing haplotypes which may link the rare haplotypes further to their respective nodes or to each other. A more intensive sampling may locate these 'missing' haplotypes. The low frequencies of the unique haplotypes probably reflect their recent origin.

Population 23 (Greece) is dominated by haplotype H5, and H3 (the most common haplotype) is absent from this population. These two haplotypes differ by a single mutation. It is probable that H5 proceeds from H3 but over the course of time H5 gained selective advantage over H3 leading to its dominance and disappearance of H3.

Haplotype diversity in Italian and Iberian populations is high with 10 different haplotypes, of which three are unique to population 19 (Italy) and another three to population 15 (Spain). On the other hand, only five haplotypes are found in northern European populations (Great Britain and Sweden), of which two are unique to population 01 (Great Britain). These two unique haplotypes (H1 and H2) could be cultivated forms of wild cherry since it was later found (Dr Richard Ennos, personal communication) that this population was an artificial population with the possibility of the occurrence of cultivated *P. avium* among the wild trees. A previous study had revealed that haplotypes of cultivars and wild *P. avium* were different (Mohanty *et al.*, 2001). Hence, the number of haplotypes in populations from Great Britain and Sweden is reduced to three. This decrease in haplotype heterogeneity from south to north of Europe is in agreement with other studies (Mohanty *et al.*, 2000; Demesure *et al.*, 1996; King & Ferris, 1998).

From the present study, it is not possible to predict any migration routes for *P. avium*. This is because one haplotype (H3) is very abundant and is common to almost all the populations; the other shared haplotypes are present at low frequency and with sporadic occurrence in populations. Furthermore, no fossil pollen data are available for this species.

The study throws light on cpDNA haplotype distribution of *P. avium* in the European deciduous forests. Genetically polymorphic populations and unique haplotypes which give special features to the populations have

been identified; this information could provide important guidelines for staging conservation and management strategies in this tree species.

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