Chloroplast DNA Diversity in *Prunus* and Its Implication on Genetic Relationships

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ABSTRACT. Chloroplast DNA (cpDNA) in 84 *Prunus* L. accessions (interspecific hybrids and *Prunus* species) were analyzed to confirm the maternal origin of the interspecific hybrids of *Prunus* and to establish genetic relationships among *Prunus* species. The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method, which included amplification of cpDNA regions with three universal primer pairs (K1K2, HK, DT) and subsequent digestion with three restriction enzymes (*AluI*, *HinfI*, *TaqI*), revealed 33 haplotypes for the 84 accessions studied. Fourteen from these cpDNA haplotypes were shared by two or more accessions, and 19 were unique. Accessions sharing the same haplotype have maternal relationships among them, which allowed identity confirmation of maternal progenitors of *Prunus* interspecific hybrids in these cases. Unweighted pair group method average (UPGMA) and minimum-length spanning tree (MST) analyses were performed based on shared common fragments and the number of mutational differences among the 33 haplotypes, respectively. The cpDNA polymorphisms detected made possible the analysis of genetic relationships among the studied *Prunus* accessions. Most of the recovered relationships are in agreement with current taxonomic hypotheses and artificial crosses.

Prunus (subfamily Prunoideae of the family Rosaceae) is a large and diverse genus comprising ≈ 400 species of trees and shrubs. This genus originated chiefly in the Northern hemisphere and is widely represented in Europe (Dosba et al., 1994). The basic chromosome number of *Prunus* is $x = 8$. Almond [*P. dulcis* D.A. Webb (formerly *P. amygdalus* Batsch)], peach [*P. persica* (L.) Batsch], apricot (*P. armeniaca* L.), and sweet cherry (*P. avium* L.) are diploids with $2n = 2x = 16$. Tetraploid species include sloe (*P. spinosa* L.), sour cherry (*P. cerasus* L.), and *P. fruticosa* Pall. ($2n = 4x = 32$) and hexaploid species are represented by European plums [*P. domestica* L. and *P. insititia* L. ($2n = 6x = 48$)]. *Prunus* is economically important because of

its diversified uses as fruit [e.g., European plums, Japanese plum (*P. salicina* Lindl.), peach, apricot, sour cherry, sweet cherry, and almond], ornamentals, forestry, or for industrial purposes (Webster, 2005). The presence of a large number of *Prunus* species increases the possibilities of interspecific hybridization and makes the establishment of botanical classification quite complicated (Layne, 1987). Traditional taxonomy of *Prunus* has used morphology to estimate relationships among the species in question and to provide taxonomy for such genus. Systematic classification in *Prunus* is still controversial. De Tournefort (1700) recognized six distinct genera within *Prunus* “*sensu lato*” based on fruit morphology: *Amygdalus* L., *Armeniaca* Mill., *Cerasus* Mill., *Laurocerasus* DuRoi, *Persica* Mill., and *Prunus* “*sensu stricto*”. Bentham and Hooker (1865) were the first to unite the six genera of De Tournefort (1700) into a single genus *Prunus*, which was subdivided into seven sections: *Amygdalopsis*, *Amygdalus*, *Armeniaca*, *Cerasoides*, *Cerasus*, *Laurocerasus*, and *Prunus*. Koehne (1893) initially divided the genus *Prunus* into seven subgenera, but later (Koehne, 1911) recognized only four: *Amygdalus*, *Cerasus*, *Padus*, and *Prunophora* (= *Prunus*). Rehder (1940) reviewed the previous treatments and divided the genus into five subgenera: *Prunus* [= *Prunophora* (Neck.)] Focke, *Amygdalus* (L.) Benth. Hook., *Cerasus* (Adans.) Focke, *Padus* (Moench) Focke, and *Laurocerasus* (Ser.) Rehd. The subgenus *Cerasus*, as defined by

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Rehder, was composed of a large and diverse group of species and was later divided by Ingram (1948) into subgenera *Cerasus* and *Lithocerasus* Ingram. At present, biochemical (isozyme) and molecular (DNA analysis) approaches allow a more accurate estimate of genetic affinities and evolutionary relatedness among *Prunus* taxa that permits a comparison with traditional classifications established by taxonomists. Several investigations were carried out to assess genetic variation among *Prunus* species using isozyme markers (Arulsekhar et al., 1986; Mowrey and Werner, 1990). Molecular markers such RFLPs, RAPDs, and SSRs were also used for phylogenetic and genetic diversity studies among *Prunus* species (Casas et al., 1999; Kaneko et al., 1986; Rohrer et al., 2004).

Noncoding regions of cpDNA have been explored under the assumption that these regions should be under less functional constraint than coding regions and should hence provide greater levels of variation for phylogenetic analyses (Gielly and Taberlet, 1994). Universal primers (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Taberlet et al., 1991) have been used to amplify fragments of noncoding regions of cpDNA followed by digestion with restriction enzymes. Fragment length polymorphism was consequently revealed within the amplified fragments (Demesure et al., 1996; El Mousadek and Petit, 1996; King and Ferris, 1998). This PCR-RFLP method, also known as cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), is a readily accessible laboratory technique that can be used to evaluate large portions of the chloroplast genome in numerous individuals in a short time and at a lower cost. This method has been used to analyze phylogenetic relationships of cultivated *Prunus* species (Badenes and Parfitt, 1995; Panda et al., 2003) and to determine the phylogeography and the population diversity in *P. spinosa* and *P. avium* (Mohanty et al., 2000, 2001, 2002).

Current trends in breeding stone fruit rootstocks are based on the production of interspecific hybrids, aiming at putting together favorable traits that appear in different species (Moreno, 2004). Commercial *Prunus* rootstocks that are a result of uncontrolled interspecific pollinations are available on the market. Moreover, the pedigree of most of the clones is unknown due to the lack of parental control, and this can be a major constraint for their use in breeding programs. It is subsequently desirable to appropriately characterize the parentage of the rootstock material as a means to evaluate the success of various crosses.

In this study, a characterization of *Prunus* species and rootstocks (interspecific hybrids) on the basis of cpDNA screening was carried out with the following objectives: 1) to analyze the genetic diversity of interspecific hybrids and species belonging to the *Prunus* genus; 2) to test the usefulness of the method to confirm the maternal origin of *Prunus* interspecific hybrids; 3) to investigate genetic relationships among *Prunus* species. The characterization of this plant material and the verification of its pedigree before it is introduced into the breeding programs of the Experimental Station of Aula Dei at Zaragoza, Spain, will be useful to increase the genetic background in these programs.

Materials and Methods

PLANT MATERIALS. Sixty-two accessions of various interspecific hybrids of the genus *Prunus* were obtained from the Krymsk Experimental Breeding Station (KEBS) in Russia

including pedigree data (see Table 1). These accessions form a group highly representative of the genetic variability in the KEBS collection. Twenty-two *Prunus* species were sampled from three important collections of *Prunus* in Europe: KEBS; Estación Experimental de Aula Dei-CSIC at Zaragoza, Spain; Institut Nationale de Recherche Agronomique (INRA) at Bordeaux and Avignon, France (Table 1). Fresh leaves were collected from plants, frozen, and stored at -80°C .

DNA EXTRACTION, AMPLIFICATION, AND DIGESTION. DNA was extracted from frozen leaves following the cetyltrimethylammonium bromide (CTAB) extraction method described by Cheng et al. (1997). DNA quality was examined by electrophoresis in 0.8% (w/v) agarose gels, and DNA concentration was quantified using a spectrophotometer. Extracted DNA was diluted to $5\text{ ng}\cdot\mu\text{L}^{-1}$ (working solution) and stored at -20°C until PCR reactions.

Initially, amplifications of cpDNA were done using five universal primer pairs (Table 2) described by Dumolin-Lapegue et al. (1997). PCR reactions were performed in a final volume of $40\text{ }\mu\text{L}$ containing 20 ng of template DNA, 0.2 mm of each of the four dNTPs, $0.2\text{ }\mu\text{M}$ of forward and reverse primers each, 2 mM MgCl_2 , and 1 U of *Pfu* DNA polymerase in the buffer provided by the manufacturers of the enzyme (Biotools, B&M Laboratories, S.A., Madrid, Spain). Amplifications were carried out in a Gene Amp 2700 thermocycler (Applied Biosystems, Foster City, CA) using the following temperature cycles: 1 cycle of 3 min at 95°C , 40 cycles of 1 min at 94°C , 45 s at $52\text{--}62^{\circ}\text{C}$, and $2\text{--}5\text{ min}$ 30 s at 72°C (annealing temperatures and extension times for each primer pair are provided in Table 2). The last cycle was followed by a final incubation for 10 min at 72°C , and then the PCR products were stored at 4°C before analysis. Amplification of cpDNA products was checked by electrophoresis on 1% (w/v) agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) light. Approximate amplified fragment sizes were estimated with a 1-kb ladder DNA marker (Invitrogen, Carlsbad, CA).

Amplified fragments were digested with three restriction enzymes: *AluI*, *HinfI*, and *TaqI* (Invitrogen). The reaction mixture was incubated overnight at 37°C with 3 U of *AluI* or *HinfI*, or 1 h at 65°C with 5 U of *TaqI*. Restriction products were separated on 2.5% (w/v) agarose gels, run in a Tris-borate-EDTA buffer (1 $\times$) for 5 h at $3\text{ V}\cdot\text{cm}^{-1}$, then stained with ethidium bromide and visualized under UV light. Restriction fragment sizes were estimated with a 50-bp ladder marker (Amersham, Piscataway, NJ).

DATA ANALYSIS. The presence or absence of each restriction fragment at each polymorphic site was scored as binary data and used to identify chloroplast haplotypes. Similarity between pairs of haplotypes was estimated from the binary matrix using the simple matching coefficient. The similarity matrix was employed to construct a dendrogram by unweighted pair group method average (UPGMA). All the analyses were computed using the programs from the NTSYS-pc package, version 2.1 (Rohlf, 2000).

Additionally, a minimum-length spanning tree between haplotypes of interspecific hybrids and *Prunus* parents was performed using the programs from the NTSYS-pc, version 2.1 (Rohlf, 2000). The procedure consists of calculating the number of mutational differences between haplotypes and then connecting them by direct links of the smallest possible length (Prim, 1957).

Table 1. List of 84 accessions (interspecific hybrids and species) of *Prunus* studied.^z

Accession	Origin ^y	Genotype (female × male)	Section ^x	Subgenus ^x	Haplotype
PACE00-03	KEBS	(<i>P. pumila</i> × <i>P. armeniaca</i>) × <i>P. domestica</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE00-08	KEBS	(<i>P. pumila</i> × <i>P. armeniaca</i>) × <i>P. domestica</i> (4x)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE01-26	KEBS	(<i>P. besseyi</i> × <i>P. salicina</i>) × (<i>P. cerasifera</i> × <i>P. persica</i>)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE01-34	KEBS	(<i>P. besseyi</i> × <i>P. armeniaca</i>) × <i>P. domestica</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE01-46	KEBS	(<i>P. pumila</i> × <i>P. armeniaca</i> × <i>P. domestica</i>)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE02-14	KEBS	(<i>P. pumila</i> × <i>P. salicina</i>) × (<i>P. cerasifera</i> × <i>P. persica</i>)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PADAC01-47	KEBS	(<i>P. pumila</i> × <i>P. armeniaca</i> × <i>P. domestica</i>)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
Drujba	KEBS	<i>P. pumila</i> × <i>P. armeniaca</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
EVRICA	KEBS	(<i>P. besseyi</i> × <i>P. salicina</i>) × <i>P. cerasifera</i> (Otletchniza)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
Krasnaia-lenta	KEBS	(<i>P. besseyi</i> × <i>P. salicina</i>) × <i>P. cerasifera</i> = Hiawatha × <i>P. cerasifera</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
Krasni bordur	KEBS	(<i>P. besseyi</i> × <i>P. salicina</i>) × <i>P. cerasifera</i> (Pissardii)	<i>Microcerasus</i>	<i>Cerasus</i>	H1
ST-N1	KEBS	<i>P. besseyi</i> × <i>P. cerasifera</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
ST-N2	KEBS	<i>P. besseyi</i> × <i>P. cerasifera</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
AD-3	EEAD	<i>P. besseyi</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H1
PACE01-35	KEBS	(<i>P. salicina</i> × <i>P. besseyi</i>) × (<i>P. cerasifera</i> × <i>P. armeniaca</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE02-05	KEBS	(<i>P. salicina</i> × <i>P. cerasifera</i>) × (<i>P. cerasifera</i> × <i>P. persica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE02-06	KEBS	(<i>P. salicina</i> × <i>P. cerasifera</i>) × (<i>P. armeniaca</i> × <i>P. persica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE02-07	KEBS	[<i>P. salicina</i> × <i>P. cerasifera</i> (4x)] × [<i>P. cerasifera</i> × <i>P. persica</i> (4x)]	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE02-08	KEBS	(<i>P. salicina</i> × <i>P. cerasifera</i>) × (<i>P. armeniaca</i> × <i>P. persica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE02-21	KEBS	[<i>P. salicina</i> (Santa Rosa) × <i>P. cerasifera</i> (4x)] × [<i>P. cerasifera</i> × <i>P. persica</i> (Kuban-86) (4x)]	<i>Euprunus</i>	<i>Prunophora</i>	H2
AD-11	EEAD	<i>P. salicina</i>	<i>Euprunus</i>	<i>Prunophora</i>	H2
PACE00-09	KEBS	<i>P. cerasifera</i> × <i>P. persica</i> (4x)	<i>Euprunus</i>	<i>Prunophora</i>	H3
PACE01-38	KEBS	<i>P. dasycarpa</i> × <i>P. domestica</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H3
PACE01-39	KEBS	<i>P. dasycarpa</i> × <i>P. spinosa</i> , F2	<i>Armeniaca</i>	<i>Prunophora</i>	H3
PACE01-40	KEBS	<i>P. dasycarpa</i> × <i>P. spinosa</i> , F2	<i>Armeniaca</i>	<i>Prunophora</i>	H3
PACE01-41	KEBS	<i>P. dasycarpa</i> × <i>P. spinosa</i> , F2	<i>Armeniaca</i>	<i>Prunophora</i>	H3
PACE02-17	KEBS	<i>P. cerasifera</i> × <i>P. persica</i> (nectarine)	<i>Euprunus</i>	<i>Prunophora</i>	H3
PACE02-18	KEBS	<i>P. cerasifera</i> × <i>P. persica</i> [Krymsk-86 (4x)]	<i>Euprunus</i>	<i>Prunophora</i>	H3
Krasnaia Kubani	KEBS	<i>P. cerasifera</i> × <i>P. persica</i> (Red leaf)	<i>Euprunus</i>	<i>Prunophora</i>	H3
Kuban-87	KEBS	<i>P. cerasifera</i> × <i>P. persica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H3
PACE01-13	KEBS	<i>P. fruticosa</i> × <i>P. mahaleb</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
PACG00-01	KEBS	<i>P. fruticosa</i> × <i>P. pennsylvanica</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
LC-52	KEBS	<i>P. cerasus</i> × <i>P. maackii</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
VSL-1	KEBS	<i>P. fruticosa</i> × <i>P. lannesiana</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
VSL-2	KEBS	<i>P. fruticosa</i> × <i>P. lannesiana</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
AD-16	INRA-B	<i>P. fruticosa</i>	<i>Eucerasus</i>	<i>Cerasus</i>	H4
AP-2	KEBS	<i>P. cerasifera</i> × <i>P. persica</i> (nectarine)	<i>Euprunus</i>	<i>Prunophora</i>	H5
Krymsk-1	KEBS	<i>P. cerasifera</i> × <i>P. tomentosa</i>	<i>Euprunus</i>	<i>Prunophora</i>	H5
PACE02-15	KEBS	<i>P. cerasifera</i> × (<i>P. communis</i> × <i>P. bucharica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H5
PACE01-42	KEBS	<i>P. kansuensis</i> × <i>P. davidiana</i> × <i>P. persica</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H6
PACE01-43	KEBS	<i>P. kansuensis</i> × <i>P. davidiana</i> × <i>P. persica</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H6
AD-19	INRA-A	<i>P. kansuensis</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H6
PACE02-10	KEBS	<i>P. brigantia</i> × <i>P. persica</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H7
PACE02-11	KEBS	<i>P. brigantia</i> × <i>P. persica</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H7
AD-20	INRA-A	<i>P. brigantia</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H7
PACE01-24	KEBS	<i>P. americana</i> × <i>P. persica</i>	<i>Prunocerasus</i>	<i>Prunophora</i>	H8
PACE02-09	KEBS	<i>P. americana</i> × <i>P. persica</i>	<i>Prunocerasus</i>	<i>Prunophora</i>	H8
PACE02-12	KEBS	<i>P. americana</i> × <i>P. persica</i>	<i>Prunocerasus</i>	<i>Prunophora</i>	H8
PACE00-02	KEBS	<i>P. tenella</i> × <i>P. dulcis</i>	<i>Chameamygdalus</i>	<i>Amygdalus</i>	H9
PACG00-02	KEBS	<i>P. nana</i> × <i>P. dulcis</i>	<i>Chameamygdalus</i>	<i>Amygdalus</i>	H9
AD-13	EEAD	<i>P. tenella</i>	<i>Chameamygdalus</i>	<i>Amygdalus</i>	H9
PACE01-30	KEBS	<i>P. cerasifera</i> × <i>P. bucharica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H10
PACE01-31	KEBS	<i>P. cerasifera</i> × <i>P. bucharica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H10
PACE01-33	KEBS	<i>P. cerasifera</i> × <i>P. kalmycovii</i>	<i>Euprunus</i>	<i>Prunophora</i>	H10
PACE00-12	KEBS	<i>P. spinosa</i> × (<i>P. cerasifera</i> × <i>P. persica</i>) (Kuban-1)	<i>Euprunus</i>	<i>Prunophora</i>	H11
PACE01-27	KEBS	<i>P. spinosa</i> × (<i>P. cerasifera</i> × <i>P. persica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H11
PACE00-07	KEBS	<i>P. spinosa</i> × <i>P. persica</i> (4x)	<i>Euprunus</i>	<i>Prunophora</i>	H11

continued next page

Table 1. Continued.

Accession	Origin ^y	Genotype (female × male)	Section ^x	Subgenus ^x	Haplotype
AD-2	EEAD	<i>P. armeniaca</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H12
AD-9	EEAD	<i>P. persica</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H13
PACEOR-03	KEBS	[(<i>P. americana</i> × <i>P. simonii</i>) × <i>P. spinosa</i>] × (<i>P. cerasifera</i> × <i>P. persica</i>)	<i>Prunocerasus</i>	<i>Prunophora</i>	H14
Vesenia plamiya	KEBS	(<i>P. americana</i> × <i>P. simonii</i>) × <i>P. cerasifera</i> (Pissardii)	<i>Prunocerasus</i>	<i>Prunophora</i>	H14
AD-18	INRA-B	<i>P. americana</i>	<i>Prunocerasus</i>	<i>Prunophora</i>	H14
PACE01-32	KEBS	<i>P. cerasifera</i> × <i>P. bucharica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H15
PACE01-28	KEBS	<i>P. cerasifera</i> × (<i>P. amygdalus</i> × <i>P. bucharica</i>)	<i>Euprunus</i>	<i>Prunophora</i>	H16
PACE02-04	KEBS	<i>P. kansuensis</i> × <i>P. persica</i> (Red leaf)	<i>Euamygdalus</i>	<i>Amygdalus</i>	H17
PACE01-37	KEBS	<i>P. cerasifera</i> × <i>P. prostrata</i> , F2	<i>Euprunus</i>	<i>Prunophora</i>	H18
PACE01-45	KEBS	<i>P. prostrata</i> × <i>P. cerasifera</i> × <i>P. domestica</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H18
PACE00-10	KEBS	<i>P. cerasifera</i> × <i>P. bucharica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H19
AD-14	EEAD	(<i>P. besseyi</i> × <i>P. salicina</i>) (Sapa)	<i>Microcerasus</i>	<i>Cerasus</i>	H20
PACE00-06	KEBS	(<i>P. besseyi</i> × <i>P. salicina</i>) (Sapa) × (<i>P. cerasifera</i> × <i>P. tomentosa</i>) (Krymsk-1)	<i>Microcerasus</i>	<i>Cerasus</i>	H20
VSV-1	KEBS	<i>P. incana</i> × <i>P. tomentosa</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H20
AD-21	EEAD	<i>P. prostrata</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H21
AD-17	EEAD	<i>P. mahaleb</i>	<i>Mahaleb</i>	<i>Cerasus</i>	H22
AD-4	EEAD	<i>P. cerasifera</i>	<i>Euprunus</i>	<i>Prunophora</i>	H23
AD-8	EEAD	<i>P. insititia</i>	<i>Euprunus</i>	<i>Prunophora</i>	H24
L-2	KEBS	<i>P. lannesiana</i>	<i>Pseudocerasus</i>	<i>Cerasus</i>	H25
AD-22	EEAD	<i>P. bucharica</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H26
AD-12	EEAD	<i>P. spinosa</i>	<i>Euprunus</i>	<i>Prunophora</i>	H27
AD-10	EEAD	<i>P. pumila</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H28
AD-7	EEAD	<i>P. incana</i>	<i>Microcerasus</i>	<i>Cerasus</i>	H29
AD-15	INRA-B	<i>P. davidiana</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H30
AD-5	EEAD	<i>P. dasycarpa</i>	<i>Armeniaca</i>	<i>Prunophora</i>	H31
AD-6	EEAD	<i>P. domestica</i>	<i>Euprunus</i>	<i>Prunophora</i>	H32
AD-1	EEAD	<i>P. dulcis</i>	<i>Euamygdalus</i>	<i>Amygdalus</i>	H33

^zClassification into sections and subgenera is according to Rehder (1940). Haplotypes found in accessions are also included.

^yKEBS, Krymsk Experimental Breeding Station, Russia; EEAD, Estación Experimental de Aula Dei-CSIC, Zaragoza, Spain; INRA-A, Institut National de Recherche Agronomique (INRA), Avignon, France; INRA-B, INRA, Bordeaux, France.

^xSection and subgenus given here correspond to the first species, representing female parent or maternal progenitor for the interspecific hybrid accessions.

Results

CHLOROPLAST DNA RESTRICTION PATTERNS. Polymorphisms in noncoding regions of the chloroplast DNA were studied in 84 interspecific hybrids and species of the genus *Prunus*. Initially, five universal primer pairs of cpDNA were used for amplification. Thereafter, and due to their high degree of amplification (Table 2), three primer pairs (DT, HK, K1K2) were chosen for this study. Estimated sizes of the fragments amplified with the

DT, HK, and K1K2 primer pairs were 1150, 1700, and 2650 bp, respectively. Total size of these fragments represent ≈4% of the total chloroplast genome (considering that cpDNA size in most *Prunus* spp. is ≈140 kbp (Kaneko et al., 1986; Uematsu et al., 1991)). No length polymorphisms were detected in the obtained PCR products using any of the primer pairs tested. Amplification products were digested by three restriction enzymes (*AluI*, *HinfI*, and *TaqI*). From the initial nine primer pair–restriction enzyme combinations, eight (DT–*AluI*, DT–*HinfI*, DT–*TaqI*, HK–*AluI*, HK–*HinfI*, K1K2–*AluI*, K1K2–*HinfI*, and K1K2–*TaqI*) gave polymorphic patterns (Table 3). The HK–*TaqI* combination showed a monomorphic pattern.

Table 2. Chloroplast DNA (cpDNA) universal primer pairs used for the amplification reactions in *Prunus* accessions; polymerase chain reaction (PCR) conditions, estimated size of amplified fragments, and quality of amplification are shown.

Abbreviation of cpDNA primers ^z	PCR conditions		Amplified fragment (bp)	Degree of amplification ^y
	Annealing temp (°C)	Extension time		
DT	52	2 min 30 s	1150	++
HK	62	3 min	1700	++
K1K2	57	4 min	2650	++
CD	57	5 min 30 s	3800	+
VL	56	5 min 30 s	3900	NA

^zAbbreviations of cpDNA primers as described by Dumolin-Lapegue et al. (1997).

^y++, Good amplification; +, weak amplification; NA, no amplification.

Table 3. Major patterns and variants (in bp) of fragments revealed in each polymorphic site detected with different primer pair–restriction enzyme combinations in 84 accessions of hybrids and parents of *Prunus* studied.

Polymorphic sites	Length or point mutations [major pattern → variant ^z (bp)]
DT–(<i>AluI</i>)1	675 (B) → 720 (A)
DT–(<i>AluI</i>)2	475 (B) → 490 (A), 460 (C), 250 + 225 (D)
DT–(<i>HinfI</i>)1	305 (B) → 315 (A), 175 + 140 (C)
DT–(<i>HinfI</i>)2	260 (C) → 275 (B), 305 (A)
DT–(<i>HinfI</i>)3	255 (A) → 250 (B)
DT–(<i>TaqI</i>)1	550 (B) → 555 (A), 540 (C)
DT–(<i>TaqI</i>)2	395 (C) → 400 (B), 375 (D), 430 (A)
DT–(<i>TaqI</i>)3	140 (B) → 150 (A)
HK–(<i>AluI</i>)1	560 (B) → 570 (A)
HK–(<i>AluI</i>)2	385 (E) → 405 (C), 375 (F), 350 (G), 415 (A), 410 (B), 400 (D), 310 (H)
HK–(<i>AluI</i>)3	65 + 45 (B) → 110 (A)
HK–(<i>HinfI</i>)1	690 (C) → 680 (D), 660 (E), 640 (F), 710 (A), 700 (B)
HK–(<i>HinfI</i>)2	240 (C) → 245 (B), 250 (A)
K1K2–(<i>AluI</i>)1	425 (A) → 420 (B)
K1K2–(<i>AluI</i>)2	330 + 130 (B) → 320 + 130 (D), 460 (A), 325 + 130 (C)
K1K2–(<i>HinfI</i>)1	230 + 255 (B) → 485 (A)
K1K2–(<i>HinfI</i>)2	380 (A) → 370 (B)
K1K2–(<i>HinfI</i>)3	340 (C) → 450 (A), 360 (B)
K1K2–(<i>HinfI</i>)4	190 (B) → 200 (A)
K1K2–(<i>TaqI</i>)1	700 (A) → 670 (B)
K1K2–(<i>TaqI</i>)2	650 (C) → 700 (A), 655 (B), 600 (D)
K1K2–(<i>TaqI</i>)3	340 (A) → 335 (B), 270 (C)
K1K2–(<i>TaqI</i>)4	265 (A) → 250 (D), 260 (B), 255 (C)

^zA, B, C, D, E, F, G, and H represent different polymorphisms in each polymorphic site.

and K1K2–*HinfI* and K1K2–*TaqI* showed four polymorphic sites (Table 3). Of the 23 polymorphic sites, 13 showed three or more patterns and the most polymorphic were HK–(*AluI*)2 and HK–(*HinfI*)1 with 8 and 6 different patterns, respectively. The rest of the polymorphic sites presented only two different restriction patterns (Table 3).

The restriction patterns obtained in the combination K1K2–*HinfI* in 11 interspecific hybrids are shown in Fig. 1. The first interspecific hybrid shows a different restriction pattern from the rest with the presence of a fragment of ≈485 bp, which is substituted by two fragments of 230 and 255 bp in the rest of the samples, incited by a point mutation (Table 3). Also, most of the interspecific hybrids show a fragment of ≈340 bp, except the fourth and the fifth ones, which both show a fragment of 450 bp and the seventh one, which shows a 360 bp fragment. This kind of polymorphism was probably due to indel mutations.

CHLOROPLAST DNA HAPLOTYPES. The combination of all the mutations resulted in 33 cpDNA haplotypes (described in Table 4), which represent the 84 accessions studied as are shown in Table 1. Fourteen from the total cpDNA haplotypes were shared by two or more accessions, and 19 were unique haplotypes to their respective accessions. Twenty-seven haplotypes were specific to only one *Prunus* species, while the remaining six (H1, H3, H4, H9, H18, and H20) were shared by two different

Fig. 1. Restriction patterns obtained on agarose gel with the primer pair–restriction enzyme combination K1K2–*HinfI* in 11 interspecific hybrids of *Prunus*; M = molecular size marker (50-bp ladder; Amersham, Piscataway, NJ). The first interspecific hybrid shows a different restriction pattern with the presence of a fragment of ≈485 bp, which is substituted by two fragments of 230 and 255 bp in the remaining samples, incited by a point mutation. Most of the interspecific hybrids show a fragment of ≈340 bp except the fourth and the fifth ones, which both show a fragment of 450 bp, and the seventh one, which shows a 360-bp fragment. This kind of polymorphism was probably due to indel mutations.

species (Table 1). On the other hand, 15 from the 24 species analyzed (directly or through their interspecific hybrids) presented only one haplotype, while eight species [*P. americana* Marshall, *P. besseyi* L.H. Bailey, *P. dasycarpa* Ehrh., *P. kansuensis* Rehd., *P. incana* (Pall.) Batsch, *P. prostrata* Labill., *P. pumila* L., and *P. spinosa*] presented two different haplotypes. *Prunus cerasifera* Ehrh. is the unique species of this study and showed eight different haplotypes (Table 1).

Haplotype H1 was the most abundant and was shared by 14 accessions (16.1%): 13 were interspecific hybrids with *P. pumila* or *P. besseyi* as female parents, and the fourteenth one was a parent representing the *P. besseyi* species (Table 1). Other hybrid accessions with *P. besseyi* as the female parent shared the haplotype H20 that had an interspecific hybrid with *P. incana* as its maternal progenitor (Table 1).

Haplotype H2 was only shared by seven accessions: six were interspecific hybrids showing *P. salicina* as the female parent, and the last one represented the species *P. salicina* itself. Similar results were found for haplotype H7, which was detected in only two interspecific hybrids with *P. brigantia* Vill. as female parent and the species *P. brigantia* itself. Moreover, haplotype H6 was detected in two interspecific hybrids with *P. kansuensis* as the female parent and the species *P. kansuensis* itself, but this species was also represented by another haplotype, H17 (Table 1). The *P. americana* accession and two interspecific hybrids with *P. americana* as female parent shared the same haplotype H14. This species also showed haplotype H8 (Table 1).

All interspecific hybrid accessions with *P. dasycarpa* as female parent shared the same haplotype H3 with some hybrid accessions presenting *P. cerasifera* as the female parent. The *P. dasycarpa* species also showed the haplotype H31. Moreover, *P. cerasifera* presented six other haplotypes (H5, H10, H15, H16, H19, and H23) and shared the haplotype H18 with *P. prostrata* (Table 1). Haplotype H4 was common to all the accessions presenting *P. fruticosa* or *P. cerasus* as the female

Table 4. Description of 33 chloroplast DNA (cpDNA) haplotypes showing mutational differences in each polymorphic site, revealed by the eight primer–restriction enzyme combinations used for analysis of diversity in the 84 *Prunus* accessions.^z

Polymorphic site	Haplotype																																	
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	
DT-(<i>AluI</i>)1	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
DT-(<i>AluI</i>)2	B	B	B	A	B	B	A	B	B	B	B	B	B	B	B	B	B	D	B	B	D	A	B	B	B	A	B	B	B	B	B	B	B	B
DT-(<i>HinfI</i>)1	B	B	B	A	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B	B	C	B	B	A	B	B	B	B	B
DT-(<i>HinfI</i>)2	C	C	C	B	C	C	C	C	C	C	C	C	B	C	C	C	C	B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
DT-(<i>HinfI</i>)3	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
DT-(<i>TaqI</i>)1	B	B	B	A	B	B	A	B	B	B	C	B	B	B	B	B	B	B	B	B	B	A	B	B	B	A	B	B	B	B	B	B	B	B
DT-(<i>TaqI</i>)2	C	C	C	C	C	C	C	C	D	C	C	C	B	C	C	B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	A	C	C
DT-(<i>TaqI</i>)3	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
HK-(<i>AluI</i>)1	B	B	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	A	B	A	A	B	B	B	B	B	B	B	B	A	A	A	B	B
HK-(<i>AluI</i>)2	C	E	E	E	E	E	F	E	E	E	C	C	E	F	E	E	E	E	A	C	A	D	E	H	C	E	E	G	E	E	E	E	H	E
HK-(<i>AluI</i>)3	B	B	B	B	B	A	B	B	B	B	B	B	A	B	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
HK-(<i>HinfI</i>)1	C	E	D	C	D	D	E	E	C	D	C	C	C	E	C	C	D	D	C	C	A	C	D	C	C	B	D	F	C	E	E	D	D	D
HK-(<i>HinfI</i>)2	B	B	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	C	B	C	C	C	C	C	C	C	C	B	C	A	C	C
K1K2-(<i>AluI</i>)1	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
K1K2-(<i>AluI</i>)2	B	B	D	B	B	B	B	A	B	B	B	B	B	B	D	D	B	B	B	B	B	B	B	B	B	B	C	B	B	B	B	B	B	C
K1K2-(<i>HinfI</i>)1	B	B	A	B	B	B	A	B	B	A	A	B	B	B	B	A	B	B	B	B	B	B	B	A	B	B	B	B	B	B	B	A	A	B
K1K2-(<i>HinfI</i>)2	A	A	B	A	A	A	A	A	A	A	A	A	A	A	B	A	B	A	A	A	A	A	A	A	A	A	B	A	A	A	A	B	A	B
K1K2-(<i>HinfI</i>)3	C	C	C	C	C	A	C	C	B	C	C	C	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
K1K2-(<i>HinfI</i>)4	B	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
K1K2-(<i>TaqI</i>)1	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
K1K2-(<i>TaqI</i>)2	C	C	A	C	C	C	C	C	D	A	B	C	C	A	A	A	C	C	C	C	C	C	C	A	A	C	C	C	C	C	C	C	C	C
K1K2-(<i>TaqI</i>)3	A	A	A	B	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
K1K2-(<i>TaqI</i>)4	A	A	D	B	A	A	A	A	A	A	A	A	A	A	D	D	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

^zA, B, C, D, E, F, G, and H refer to different patterns of each polymorphic site (see Table 3 for codes).

parent and to the species *P. fruticosa* itself. The *P. tenella* Batsch accession and two interspecific hybrids with *P. tenella* or *P. nana* (L.) Stokes as female parent were included under the same haplotype, H9.

Chloroplast DNA diversity and genetic relationships in *Prunus*

UPGMA ANALYSIS. The genetic diversity among the different cpDNA haplotypes was shown in the UPGMA cluster analysis. At a similarity coefficient value of 0.82, the phenogram showed two main groups, and three haplotypes (H9, H11, and H27) appeared out of the groups (Fig. 2).

The first main group comprised haplotypes of *Prunus* species belonging to the subgenera *Prunophora*, *Amygdalus*, and *Cerasus* section *Microcerasus* Webb. The second main

group was clustered to the first one at a similarity value of 0.77 and was composed of four haplotypes, three representing species of the subgenus *Cerasus* section *Eucerasus* Koehne (H4), section *Mahaleb* Focke (H22), and section *Pseudocerasus* Koehne (H25), and the fourth haplotype (H7) was present in *P. brigantiaca* species, member of subgenus *Prunophora* section *Armeniaca* (Mill.) Koch. Two of the three haplotypes clustered out of the groups were represented by *P. spinosa* species (H27 and H11), while the third one (H9) represented the cpDNA type of *P. tenella* and *P. nana* species.

In the first main group, three subgroups were observed (Fig. 2). A close relationship was detected among haplotypes included in the first subgroup, representing species of the subgenus *Prunophora* sections *Armeniaca*, *Euprunus* Koehne,

Fig. 2. Dendrogram of 33 *Prunus* chloroplast DNA (cpDNA) haplotypes (H1-H33, described in Table 4) generated by UPGMA clustering analysis using the simple matching coefficient. Classification into subgenera and sections is according to Rehder (1940).

and *Prunocerasus* Koehne, and species of the subgenus *Cerasus* section *Microcerasus* (H1, H20, H21, H28, and H29; see Table 1). Haplotypes representing species of the subgenus *Amygdalus* section *Euamygdalus* Spach. were clustered together under the second subgroup (Fig. 2). The third subgroup, clustered at a similarity value of 0.82 from the previous two, contained haplotypes, which mostly represented species of subgenus *Prunophora* section *Euprunus*. Haplotypes representing species of section *Armeniaca* (H3 and H31) were also included in this subgroup.

MINIMUM-LENGTH SPANNING TREE ANALYSIS. The relationships among the 33 cpDNA haplotypes were also presented in a minimum-length spanning tree (MST) shown in Fig. 3. The MST offers a good representation of linked haplotype groups based on the mutational differences among them. Two main clusters of haplotypes could be observed with H23 and H5 as the internal nodes. The cluster linked to H23 contained haplotypes representing species of subgenus *Prunophora* section *Euprunus* and section *Armeniaca* for *P. dasycarpa* (H3, H31) species. The cluster linked to H5 was composed of haplotypes representing species of subgenera *Amygdalus*, *Cerasus*, and *Prunophora*.

Discussion

CHLOROPLAST DNA HAPLOTYPES: INHERITANCE AND RELATIONSHIPS. Assessment of cpDNA variation in 84 accessions representing 62 interspecific hybrids and 22 species of *Prunus* revealed 33 different cpDNA haplotypes. Twenty-seven from the total haplotypes observed were specific to a unique species (Table 1), which is very interesting for further molecular characterization of *Prunus* species. Considering that chloroplast DNA is maternally inherited in most angiosperms, assessment of cpDNA variation in *Prunus* accessions provides good information on their maternal relationships.

Haplotype H2 was shared by the species *P. salicina* and six complex interspecific hybrids of *Prunus* which have *P. salicina* as maternal progenitor (Table 1). The same case was observed for some interspecific hybrids represented by other six haplotypes (H1, H4, H6, H7, H9, and H14), which had the same haplotypes as their maternal progenitors (Table 1). These results seem to agree with the maternal inheritance of cpDNA in *Prunus* and, in some cases, permit identity confirmation of the maternal progenitor. However, some interspecific hybrids showed different haplotypes from the expected maternal ones. This fact was observed when the *Prunus* species analyzed directly or through their interspecific hybrids presented two or more different haplotypes, as *P. americana*, *P. besseyi*, *P. dasycarpa*, *P. kansuensis*, *P. incana*, *P. prostrata*, *P. pumila*, *P. spinosa*, and *P. cerasifera*. This last species is the only one in this study that showed eight different haplotypes, probably due to the large number of *P. cerasifera* accessions (16) analyzed (directly or through their interspecific hybrids) (Table 1). The mentioned discordances could be due to the existence of

Fig. 3. Minimum-length spanning tree of 33 chloroplast DNA (cpDNA) haplotypes (H1 to H33, described in Table 4) from the 84 *Prunus* accessions studied.

intraspecific cpDNA variation, which has been previously reported in *Prunus* species such *P. spinosa* and *P. avium* (Mohanty et al., 2000, 2001, 2002). Therefore, to confirm the maternal origin of interspecific hybrids based on their cpDNA haplotypes, it will be necessary to analyze more accessions of these *Prunus* species to determine better their intraspecific cpDNA diversity.

Several pairs of *Prunus* species shared the same cpDNA haplotypes (H1, H3, H4, H9, H18, and H20; Table 1). The predominant haplotype H1 was presented in the *P. besseyi* species accession and in 13 hybrids of *Prunus* having *P. pumila* or *P. besseyi* as female parent (Table 1). If it is assumed that cpDNA is maternally inherited in *Prunus*, then the obtained result provides information about the existence of a maternal relationship between *P. pumila* and *P. besseyi*. In fact, these two species are members of the same subgenus *Cerasus* section *Microcerasus* as defined by Rehder (1940). Close relationships between *P. pumila* and *P. besseyi* were also reported by Mowrey and Werner (1990) using isozyme markers.

On the other hand, nine interspecific hybrid accessions presented in common the haplotype H3; these hybrids have *P. cerasifera* or *P. dasycarpa* as female parents. This result would indicate a close maternal relationship between these two species. *Prunus dasycarpa* is, indeed, a naturally occurring hybrid of *P. cerasifera* and *P. armeniaca* (Mehlenbacher et al., 1990). Moreover, these authors reported that *P. cerasifera* × *P. armeniaca* produces hybrids resembling the natural interspecific hybrid species *P. dasycarpa* when plum is used as the female parent. According to these findings and the results obtained in this study, we can suggest that *P. cerasifera* is the maternal progenitor of *P. dasycarpa*.

Haplotype H4 was shared by six accessions: five are hybrids which have *P. fruticosa* or *P. cerasus* as female parents, and one represents the *P. fruticosa* itself. The grouping of these six accessions under the same haplotype H4 could be due to the existence of close maternal relationships between *P. fruticosa* and *P. cerasus*. In fact, these two species belong to the same subgenus *Cerasus* section *Eucerasus*. In addition, sour cherry (*P. cerasus*) is considered to be the product of interspecific hybridization of sweet cherry (*P. avium*) and ground cherry (*P. fruticosa*) (Olden and Nybom, 1968). According to Mehlenbacher et al. (1990), crossings between ground cherry and diploid or tetraploid sweet cherry selections gave progenies

resembling sour cherry. Moreover, the study of Panda et al. (2003) confirms that *P. avium* is not the maternal parent of *P. cerasus*. Our findings are in agreement with these reports, suggesting that *P. fruticosa* may be the female parent of *P. cerasus*.

Haplotype H9 was shared by three accessions: two are hybrids having *P. tenella* or *P. nana* as female parent, and one represents the species *P. tenella* (Table 1). According to Rehder (1940), *P. tenella* and *P. nana* are synonyms, which can explain their cpDNA pattern similarity.

CHLOROPLAST DNA DIVERSITY AND GENETIC RELATIONSHIPS IN *PRUNUS*. The cpDNA data obtained from the PCR-RFLP analysis of the 84 *Prunus* accessions were used to produce a dendrogram by the UPGMA cluster method and a MST. Comparison of the cpDNA phenogram with the taxonomic classification of *Prunus* based on morphological data (Rehder, 1940) shows similarities but also several apparent discrepancies.

In the UPGMA phenogram (Fig. 2), the first main group contained all haplotypes representing species of subgenus *Cerasus* section *Microcerasus*, which clustered with haplotypes representing species of subgenera *Prunophora* and *Amygdalus*. The remaining haplotypes representing species of subgenus *Cerasus*, sections *Eucerasus* (H4), *Mahaleb* (H22) and *Pseudocerasus* (H25), were clustered in the second main group. Therefore, the traditional classification of *Microcerasus* included within subgenus *Cerasus* (Rehder, 1940) was not supported by this UPGMA analysis. Moreover, Bortiri et al. (2001) and Lee and Wen (2001) also noted that section *Microcerasus* does not show any relationship to the rest of the subgenus *Cerasus*. Likewise, Katayama and Uematsu (2005) found that *Amygdalus*, *Prunophora*, and *Cerasus* section *Microcerasus* formed a major group in the UPGMA tree performed using RFLP data from chloroplast DNA. A similar relation among haplotypes representing *Microcerasus* species and haplotypes representing the rest of species of the subgenus *Cerasus* was also shown in the MST analysis (Fig. 3).

The first main group in the UPGMA phenogram was composed of three subgroups (Fig. 2). The first subgroup included species of section *Microcerasus* and species of subgenus *Prunophora*. This subgroup showed the close relatedness of *P. pumila* and *P. besseyi* (H1, H20, and H28) to plums [*P. salicina* and *P. cerasifera* (H2, H5, and H19)], and to apricot [*P. armeniaca* (H12)]. Rammings and Cociu (1990) reported that the cherry species *P. besseyi* and *P. pumila* appeared more closely related to plums than to cherries. Bortiri et al. (2001) mentioned that section *Microcerasus* was not monophyletic and that most of its species might be more closely related to the subgenus *Prunophora* than to subgenus *Cerasus*. More research is required to find the closest relatives belonging to *Microcerasus* species.

Haplotypes representing species of the subgenus *Amygdalus* section *Euamygdalus* were grouped together in the second subgroup (Fig. 2). The *P. kansuensis*, *P. persica*, and *P. davidiana* (Carriere) Franch. peach species examined in this study, and represented by haplotypes H6, H17, H13, and H30 (Table 1), clustered together at a high similarity value (0.86). The *P. bucharica* (Korsch.) Hand-Mazz. (H26) and *P. dulcis* (H33) almond species were closely related at a similarity value of 0.94, and they were also joined to peach species. In fact, members of section *Euamygdalus* were often classified as either almond-like or peach-like plants (Scorza and Okie, 1990), which is well supported by our findings. The MST (Fig. 3) also showed close relationships among *Amygdalus* haplotypes

(section *Euamygdalus*) and was in agreement with the UPGMA clustering. Nevertheless, the *P. tenella* member of subgenus *Amygdalus* section *Chameamygdalus* Spach., represented by haplotype H9, appears out of the *Amygdalus* group (Figs. 2 and 3). This result indicates that the cpDNA of *P. tenella* can be highly divergent from the other *Amygdalus* species. Kester et al. (1990) reported that the section *Chameamygdalus* includes species genetically divergent from species of other almond sections and that it rarely crosses with cultivated almond.

The close relationships showed between H24 (*P. insititia*) and H32 (*P. domestica*) (Fig. 2) was also reported by Bouhadida et al. (2005) and Casas et al. (1999) analyzing the genetic diversity of *Prunus* rootstocks with SSR and RAPD markers, respectively. These two species were closely related to *P. cerasifera*, represented by H10 and H23 (Figs. 2 and 3). *Prunus domestica* was thought to be derived from a crossing between a diploid ($2n = 2x = 16$) cherry plum or myrobalan, *P. cerasifera*, and a tetraploid ($2n = 4x = 32$) sloe or blackthorn, *P. spinosa* (Crane and Lawrence, 1952). This hypothesis has been widely accepted by horticulturists and crop plant evolutionists. However, Zohary (1992) reported that *P. domestica* probably evolved directly from the variable *P. cerasifera*, because of the close morphological similarities between them, and the wide morphological divergence between these two plums and *P. spinosa*. According to the UPGMA dendrogram and the MST analyses (Figs. 2 and 3), haplotypes H11 and H27 representing the *P. spinosa* plum species appear separated from haplotypes representing other plum species.

Prunus fruticosa and *P. cerasus* species (haplotype H4) were grouped together with *P. brigantia* (H7), *P. mahaleb* (Dougl.) L. (H22), and *P. lannesiana* (Carr.) E.H. Wilson (H25) (Figs. 2 and 3). An apparent common maternal inheritance may exist among these species. This result can be explained by the fact that all species of this group belong to the subgenus *Cerasus* with the exception of *P. brigantia*, which belong to subgenus *Prunophora* section *Armeniaca*. According to the phylogenetic studies of Mowrey and Werner (1990) based on isozymes, *P. mahaleb* a member of the section *Mahaleb* was clustered together with *P. cerasus* and *P. fruticosa*, which were classified within section *Eucerasus*. Bortiri et al. (2001) reported in their nuclear ITS and chloroplast DNA study that *P. lannesiana* was a synonym of *P. serrulata* Lindl. and that these species was closely related to *P. fruticosa* and *P. cerasus*. Our results are in agreement with these findings. The placement of European native apricot species *P. brigantia* (H7) within this group obscured its relationship with respect to other apricots. Bortiri et al. (2001) also reported that *P. brigantia* was not included in the *Armeniaca* clade, and that the ITS sequence of this species had many ambiguities, some of them in regions of high variability. Additional studies including more accessions of *Prunus* species and using more molecular data (e.g., more combinations of primers and restriction enzymes) would be required to understand the true position of *P. brigantia* within the genus *Prunus* and to draw a more accurate phylogeny of *Prunus*.

In summary, the results of this work demonstrate the usefulness of cpDNA diversity detected using PCR-RFLP methods to analyze the genetic relationships among species and cultivars of *Prunus*. This study contributes to the understanding of the maternal inheritance of cpDNA in *Prunus*, and permits confirmation of the maternal progenitors of different hybrids of this genus. The maternal inheritance of cpDNA and

the genetic relationships revealed by the cpDNA variation will be a useful tool in designing crosses for future breeding programs. The cpDNA analysis will also provide a starting point for a more detailed phylogenetic analysis of the genus *Prunus*.

Literature Cited

- Arulsekhar, S., D.E. Parfitt, and D.E. Kester. 1986. Comparison of isozyme variability in peach and almonds cultivars. *J. Hered.* 77:272–274.
- Badenes, M.L. and D.E. Parfitt. 1995. Phylogenetic relationships of cultivated *Prunus* species from an analysis of chloroplast DNA variation. *Theor. Appl. Genet.* 90:1035–1041.
- Bentham, G. and J.D. Hooker. 1865. *Genera plantarum*, Vol. 1. Reeve & Co., London.
- Bortiri, E., S.H. Oh, J. Jiang, S. Baggett, A. Granger, C. Weeks, M. Buckingham, D. Potter, and D.E. Parfitt. 2001. Phylogeny and systematics of *Prunus* (Rosaceae) as determined by sequence analysis of ITS and the chloroplast *trnL-trnF* spacer DNA. *Syst. Bot.* 26:797–807.
- Bouhadida, M., A.M. Casas, M.A. Moreno, and Y. Gogorcena. 2005. Genetic diversity of *Prunus* rootstocks using microsatellite markers. *Acta Hort.* 663:625–627.
- Casas, A.M., E. Igartua, G. Balaguer, and M.A. Moreno. 1999. Genetic diversity of *Prunus* rootstocks analysed by RAPD markers. *Euphytica* 110:139–149.
- Cheng, F.S., S.K. Brown, and N.F. Weeden. 1997. A DNA extraction protocol from various tissues in woody species. *HortScience* 32: 921–922.
- Crane, M.B. and W.J.C. Lawrence. 1952. *The genetics of garden plants*. 4th ed. Macmillan, London.
- Demesure, B., N. Sodji, and R.J. Petit. 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., B. Comps, and R.J. Petit. 1996. Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. *Evolution Int. J. Org. Evolution* 50:2515–2520.
- De Tournefort, J.P. 1700. *Institutiones Rei Herbariae*. Editio Altera, Gallica Longe Auctior, Quingentis Circiter Tabulis Aeneis Adornata, Typographia Regia, Paris.
- Dosba, F., R. Bernhardt, and A. Zanetto. 1994. Importance des ressources génétiques des *Prunus*. *C.R. Acad. Agr. France* 80:45–57.
- Dumolin-Lapegue, S., M.H. Pemonge, and R.J. Petit. 1997. An enlarged set of consensus primers for the study of organelle DNA in plants. *Mol. Ecol.* 6:393–397.
- El Mousadek, A. and R.J. Petit. 1996. Chloroplast DNA phylogeography of the argan tree of Morocco. *Mol. Ecol.* 5:547–555.
- Gielly, L. and P. Taberlet. 1994. The use of chloroplast DNA to resolve plant phylogenies: non-coding vs RbcL sequences. *Mol. Biol. Evol.* 11:769–777.
- Ingram, C. 1948. *Ornamental cherries*. Country Life, London.
- Kaneko, T., T. Terachi, and K. Tsunewaki. 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.
- Katayama, H. and C. Uematsu. 2005. Structural analysis of chloroplast DNA in *Prunus* (Rosaceae): evolution, genetic diversity and unequal mutations. *Theor. Appl. Genet.* 111:1430–1439.
- Kester, D.E., T.M. Gradziel, and C. Grasselly. 1990. Almonds (*Prunus*), p. 699–758. In: J.N. Moore and J.R. Ballington (eds.). *Genetic resources of temperate fruit and nut crops*. International Society for Horticultural Science, Wageningen, The Netherlands.
- King, R.A. and C. Ferris. 1998. Chloroplast DNA phylogeography of *Alnus glutinosa* (L.). *Gaertn. Mol. Ecol.* 7:1151–1161.
- Koehne, E. 1893. *Deutsche Dendrologie*. Verlag von Ferdinand Enke, Stuttgart, Germany.
- Koehne, E. 1911. Die Gliederung von *Prunus* subgen. *Padus*, Vol. 52, pp. 101–108. *Verhandlungen des Botanischen Vereins der Provinz Brandenburg*. Der Botanische Verein der Provinz Brandenburg, Berlin.
- Konieczny, A. and F.M. Ausubel. 1993. A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific PCR-based markers. *Plant J.* 4:403–410.
- Layne, R.E.C. 1987. Peach rootstocks, p. 185–216. In: R.C. Rom and R.F. Carlson (eds.). *Rootstocks for fruit crops*. Wiley, New York.
- Lee, S. and J. Wen. 2001. A phylogenetic analysis of *Prunus* and the *Amygdaloideae* (Rosaceae) using ITS sequences of nuclear ribosomal DNA. *Amer. J. Bot.* 88:150–160.
- Mehlenbacher, S.A., V. Cociu, and L.F. Hough. 1990. Apricots (*Prunus*), p. 63–173. In: J.N. Moore and J.R. Ballington (eds.). *Genetic resources of temperate fruit and nut crops*. International Society for Horticultural Science, Wageningen, The Netherlands.
- Mohanty, A., J.P. Martin, and I. Aguinalalde. 2000. Chloroplast DNA diversity within and among populations of the allotetraploid *Prunus spinosa* L. *Theor. Appl. Genet.* 100:1304–1310.
- Mohanty, A., J.P. Martin, and I. Aguinalalde. 2001. A population genetic analysis of chloroplast DNA in wild populations of *Prunus avium* L. in Europe. *Heredity* 87:421–427.
- Mohanty, A., J.P. Martin and I. Aguinalalde. 2002. Population genetic analysis of European *Prunus spinosa* (Rosaceae) using chloroplast DNA markers. *Amer. J. Bot.* 89:1223–1228.
- Moreno, M.A. 2004. Breeding and selection of *Prunus* rootstocks at the Aula Dei Experimental Station, Zaragoza, Spain. *Acta Hort.* 658:519–528.
- Mowrey, B.D. and D.J. Werner. 1990. Phylogenetic relationships among species of *Prunus* as inferred by isozyme markers. *Theor. Appl. Genet.* 80:129–133.
- Olden, E.J. and N. Nybom. 1968. On the origin of *Prunus cerasus* L. *Hereditas* 59:327–345.
- Panda, S., J.P. Martin, and I. Aguinalalde. 2003. Chloroplast DNA study in sweet cherry cultivars (*Prunus avium* L.) using PCR-RFLP method. *Genet. Resour. Crop Evol.* 50:489–495.
- Prim, R.C. 1957. Shortest connection networks and some generalizations. *Bell Laboratories Tech. J.* 36:1389–1401.
- Ramming, D.W. and V. Cociu. 1990. Plums (*Prunus*), p. 233–287. In: J.N. Moore and J.R. Ballington (eds.). *Genetic resources of temperate fruit and nut crops*. International Society for Horticultural Science, Wageningen, The Netherlands.
- Rehder, A. 1940. *A manual of cultivated trees and shrubs hardy in North American exclusive of the subtropical and warmer temperate regions*. 2nd ed. Macmillan, New York.
- Rohlf, F.J. 2000. *NTSYS-pc numerical taxonomy and multivariate analysis system*. Version 2.1. Exeter Software, Setauket, NY.
- Rohrer, J.R., R. Ahmad, S.M. Southwick, and D. Potter. 2004. Microsatellite analysis of relationships among North American plums (*Prunus* sect. *Prunocerasus*, Rosaceae). *Plant Syst. Evol.* 244:69–75.
- Scorza, R. and W.R. Okie. 1990. Peaches (*Prunus*), p. 175–231. In: J.N. Moore and J.R. Ballington (eds.). *Genetic resources of temperate fruit and nut crops*. International Society for Horticultural Science, Wageningen, The Netherlands.
- Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* 17:1105–1109.
- Uematsu, C., T. Sasakuma, and Y. Ogihara. 1991. Phylogenetic relationships in the stone fruit group of *Prunus* as revealed by restriction fragment analysis of chloroplast DNA. *Jpn. J. Genet.* 66:59–69.
- Webster, A.D. 2005. The origin, distribution and genetic diversity of temperate tree fruits, p. 1–11. In: J. Tromp, A.D. Webster, and S.J. Wertheim (eds.). *Fundamentals of temperate zone tree fruit production*. Backhuys Publishers, Leiden, The Netherlands.
- Zohary, D. 1992. Is the European plum, *Prunus domestica* L., a *P. cerasifera* Ehrh. × *P. spinosa* L. allo-polyploid? *Euphytica* 60: 75–77.