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Análisis de la diversidad genética en melocotonero [*P. persica* (L.) Batsch] y en patrones del género *Prunus*

MARIEM BOUHADIDA

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ANÁLISIS DE LA DIVERSIDAD GENÉTICA EN MELOCOTONERO [*P. persica* (L.) Batsch] Y EN PATRONES DEL GÉNERO *Prunus*

TESIS DOCTORAL

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Tesis Doctoral

Análisis de la diversidad genética en melocotonero [*P. persica* (L.) Batsch] y en patrones del género *Prunus*

Memoria presentada por Doña. Mariem Bouhadida, Ingeniero Agrónomo especialidad: Producción Vegetal, para optar al grado de Doctor en Ciencias

Zaragoza, Marzo 2007

Dña. Mª YOLANDA GOGORCENA AOIZ y Dña. Mª ÁNGELES MORENO SÁNCHEZ, Científicos Titulares del Consejo Superior de Investigaciones Científicas

CERTIFICAN

Que la Tesis Doctoral titulada "**Análisis de la diversidad genética en melocotonero [*P. persica* (L.) Batsch] y en patrones del género *Prunus***", ha sido realizada por Doña. MARIEM BOUHADIDA, en el Departamento de Pomología de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor en Ciencias.

Zaragoza, Marzo de 2007

Fdo. Mª Yolanda Gogorcena Aoiz

Fdo. Mª Ángeles Moreno Sánchez

A mi hijo Aymen
A mi marido, por su amor, su paciencia y su apoyo incondicional
A mi familia

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RESUMEN

El género *Prunus* pertenece a la familia de las Rosáceas (subfamilia *Prunoideae*) e incluye numerosas especies. Este género incluye diversas especies frutales de hueso de gran importancia económica como albaricoquero, almendro, ciruelo, cerezo, melocotonero, así como especies ornamentales.

El gran número de especies *Prunus* existentes aumenta significativamente las posibilidades de hibridación ínterespecífica entre ellas, por lo que se dificulta una correcta clasificación botánica. Por otra parte, los programas de mejora en patrones de frutales de hueso se basan en la producción de híbridos ínterespecíficos, con el objetivo de reunir en el mismo individuo características de interés de cada especie. Los programas de mejora genética dependen de la caracterización correcta del material vegetal para el control y la utilización eficiente de los patrones y variedades frutales de *Prunus*.

El melocotonero (*P. persica*) es uno de los frutales más tecnificado y más extendido en todo el mundo. El melocotonero es la especie con mayor dinamismo varietal dentro de los frutales del género *Prunus*. La caracterización y la identificación correcta de los cultivares es importante para la mejora y certificación, así como para la protección de los derechos de los obtentores en el mundo. En España, las variedades cultivadas tradicionalmente han sido reemplazadas por las nuevas selecciones de los programas de mejora, reduciendo drásticamente la diversidad en melocotonero. La caracterización molecular del germoplasma local mediante los marcadores moleculares es necesaria para catalogar los recursos fitogenéticos y conservar así la biodiversidad disponible en esta especie.

En este trabajo, se han caracterizado 30 cultivares de melocotonero procedentes de un mismo área de cultivo en Aragón (19 de los 30 cultivares son supuestos clones de una misma variedad: ‘Miraflores’), utilizando 20 marcadores microsatélites (SSRs) desarrollados en melocotonero. Todos los marcadores amplificaron un valor medio de 2,3 alelos por *locus*, y permitieron la identificación de 16 genotipos de los 30 estudiados, mientras que 14 clones de ‘Miraflores’ mostraron el mismo perfil genético para todos los *loci*.

Por otra parte, se ha caracterizado un conjunto de 94 cultivares de melocotonero pertenecientes al banco de germoplasma español, de referencia nacional de melocotonero, que incluye 64 cultivares locales españoles y 30 cultivares procedentes de programas de mejora internacionales. Se eligieron 15 marcadores SSRs, seleccionados por su alto nivel de polimorfismo en melocotonero. Se obtuvo un valor medio de 6,7 alelos por *locus* con todos los marcadores, mostrando así una alta diversidad genética entre los 94 cultivares estudiados. Se ha observado una mayor distancia genética entre los melocotones de carne dura y los melocotones de carne blanda, y principalmente entre los melocotones españoles de carne dura y el resto de los cultivares. Con sólo 8 de los 15 SSRs utilizados en este trabajo, se pueden distinguir correctamente todos los cultivares estudiados.

En este estudio, se han caracterizado mediante los marcadores SSRs y la PCR-RFLP, especies y patrones (híbridos ínterespecíficos) de *Prunus*, basándose en el análisis del ADN nuclear y cloroplástico, respectivamente.

Se llevó a cabo, con veinte SSRs, la caracterización de 44 patrones de *Prunus* clasificados en tres grupos: híbridos de melocotonero y almendro x melocotonero; ciruelos de crecimiento rápido; y ciruelos de crecimiento lento. Los SSRs utilizados, han mostrado un alto nivel de polimorfismo y amplificaron 16 alelos por *locus*, permitiendo la identificación total de todos los patrones estudiados.

Finalmente, la técnica de PCR-RFLP se ha utilizado para el análisis del ADN cloroplástico de 84 accesiones de *Prunus* (híbridos ínterespecíficos y especies de *Prunus*). Esta técnica se ha basado, en este estudio, en la combinación de amplificaciones mediante cebadores universales (DT, HK, K1K2) y digestiones con enzimas de restricción (*Alu*I, *Hinf*I, y *Taq*I). El polimorfismo generado en el tamaño de los fragmentos de restricción, ha permitido la detección de 33 haplotipos para las 84 accesiones estudiadas. Las accesiones que comparten el mismo haplotípo tienen relaciones maternas entre sí, lo que ha permitido en algunos casos, comprobar la identidad de los progenitores maternos de los híbridos ínterespecíficos. En este trabajo, se discuten las relaciones filogenéticas entre las especies de *Prunus* estudiadas, basándose en el análisis de los 33 haplotipos encontrados, permitiendo confirmar algunas hipótesis botánicas anteriores.

SUMMARY

Prunus (subfamily *Prunoideae* of the family *Rosaceae*) is a large and diverse genus comprised of a large species of trees and shrubs. *Prunus* is economically important because of its diversified use, as fruit (e.g., almonds, apricots, cherries, peaches, and plums), ornamentals, and forestry or industrial purposes. The presence of large number of *Prunus* species increase the possibilities of interspecific hybridisation, and make the establishment of botanical classification quite complicated. On the other hand, current trends in breeding stone fruit rootstocks are based on the production of interspecific hybrids, aiming at putting together favourable traits which appear in different species. Effective control and utilisation of *Prunus* rootstocks in breeding programs and *Prunus* management depends upon accurate and unambiguous characterisation of the plant material.

Peach is one of the most deciduous fruit tree species of the *Prunus* genus. It has a dynamic process of development and selection of new varieties. Therefore, precise cultivar identification and characterisation is essential for improving and securing peach culture in the world. On the other hand, the diversity of peach germplasm has been drastically reduced in Spain and in the world by the wide adoption of improved varieties reducing genetic resources. A detailed characterization of the Spanish peach germplasm using microsatellite markers is necessary to conserve these important genetic resources and to establish an efficient collection.

In this study, we characterised 30 peach cultivars originated from a local area in Aragón (Spain) (19 are described as clones of the 'Miraflores' peach variety) using 20 SSR primer pairs developed in peach. All markers amplified an average of 2.3 alleles per locus, and identified 16 genotypes from the 30 studied, while 14 (clones of 'Miraflores) showed the same genetic profile.

Moreover, we characterised a sample of 94 peach cultivars from the national collection composed of 64 local Spanish peach and 30 cultivars from modern breeding programs. For this aim, we used a set of 15 microsatellite markers selected for their high polymorphism from published sequences. An average of 6.7 alleles per locus was amplified with all SSRs, expressing a high genetic diversity within the 94 cultivars studied. High genetic distances were observed when comparing non-melting peach cultivars with melting ones, and especially between Spanish non-melting peaches and the rest of cultivars. Only eight SSRs from the 15 used in this study, were able to distinguish unambiguously all the cultivars studied.

In this study, a characterisation of *Prunus* species and rootstocks (interspecific hybrids) was also carried out, on the basis of nuclear and chloroplast DNA analyses using SSRs and the PCR-RFLP, respectively.

A set of 20 SSRs was used to characterize 44 *Prunus* rootstocks belonging to three groups: Peach-based rootstocks; Myrobalan-Marianna plums; and Slow growing plums. SSRs in this case showed a high level of polymorphism and amplified an average of 16 alleles per locus, allowing the total identification of all rootstocks.

The PCR-RFLP technique was used for the cpDNA screening of 84 *Prunus* accessions (interspecific hybrids and *Prunus* species) and basing on the combination of amplifications with 3 universal primers (DT, HK, and K1K2) and digestions with 3 restriction enzymes (*Alu*I, *Hinf*I, and *Taq*I). The restriction fragment size polymorphisms detected in this case revealed a total of 33 haplotypes for all the 84 accessions studied. Accessions sharing the same haplotype had maternal relationships among them, which allowed the identification of some maternal progenitors of the *Prunus* interspecific hybrids. The 33 haplotypes found in this study, enabled the reconstruction and the discussion of the phylogenetic relationships among the *Prunus* species studied.

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CAPÍTULO 1

Introducción general

1-1. EL GÉNERO *Prunus*

El género *Prunus* pertenece a la familia *Rosaceae* (subfamilia *Prunoideae*) e incluye especies de gran importancia económica como el almendro (*P. dulcis* D.A. Webb), el melocotonero [*P. persica* (L.) Batsch], el albaricoquero (*P. armeniaca* L.), y el cerezo (*P. avium* L.), etc. Estas especies son diploides, con un número básico de cromosomas x=8 y 2n=16. El ciruelo incluye diferentes especies *Prunus*, tanto diploides (*P. cerasifera* Ehrh.) como tetraploides (*P. spinosa* L.) y hexaploides (*P. domestica* L. y *P. insititia* L.), todas ellas incluidas dentro del subgénero *Prunophora*.

Existe una gran variabilidad genética y morfológica entre las distintas especies del género *Prunus*. La mayoría de ellas tienen su origen en las zonas con clima templado del hemisferio norte (Dosba et al., 1994) y fueron trasladadas de un continente a otro, a través de las rutas comerciales. El gran número de especies existentes aumenta significativamente las posibilidades de hibridación ínterespecífica entre ellas, lo que complica el establecimiento de una correcta clasificación botánica. Dentro de la familia *Rosaceae*, los botánicos han propuesto numerosas clasificaciones, basándose en las características morfológicas, proponiendo desde 70 (Krüssman, 1976) a 400 (Rehder, 1940) especies distintas dentro del género *Prunus*. La taxonomía del género *Prunus* se ha basado sobre todo en el fenotipo, estimando las diferencias genéticas entre especies. Sin embargo, la clasificación filogenética en *Prunus* es todavía controvertida. De Tournefort (1700) identificó seis géneros distintos dentro del *Prunus* s.l. (sensu lato) basándose en la morfología: *Amygdalus* L., *Armeniaca* Miller, *Cerasus* Miller, *Laurocerasus* Duhamel, *Persica* Miller, y *Prunus* s.s. (sensu stricto). Posteriormente, Bentham y Hooker (1865) unificaron los seis géneros en un único género *Prunus*, que se dividió en siete secciones: *Amygdalopsis*, *Amygdalus*, *Armeniaca*, *Cerasoides*, *Cerasus*, *Laurocerasus* y *Prunus*. Posteriormente, Koehne (1893) dividió el género *Prunus* en siete subgéneros, reduciéndolos posteriormente (Koehne, 1911) a cuatro: *Amygdalus*, *Cerasus*, *Padus* y *Prunophora* (=*Prunus*). Rehder (1940) revisó la clasificación propuesta por Koehne y dividió el género en los cinco subgéneros descritos en la Tabla 1-1: *Prunus* [= *Prunophora* (Neck.) Focke]; *Amygdalus* (L.) Benth. Hook., *Cerasus* (Adans.) Focke; *Padus* (Moench) Focke; y *Laurocerasus* (Ser.) Rehd. El subgénero *Cerasus*, definido por Rehder, está compuesto de una amplia y diversa gama de especies, y por ello se dividió en dos subgéneros: *Cerasus* y

Lithocerasus Ingram (Ingram, 1948). No obstante, la taxonomía propuesta por Rehder (1940) ha sido la más aceptada hasta la actualidad.

Tabla 1-1. *Clasificación taxonómica según Rehder (1940).*

Género	Subgénero	Sección	Especies
Prunus	***Prunus* = *Prunophora* (Neck.) Focke**	***Euprunus***	*P. cerasifera, P. domestica, P. insititia, P. salicina, P. simonii,...*
		Prunocerasus	*P. americana, P. hortulana, P. munsoniana, P. subcordata,…*
		Armeniaca	*P. armeniaca, P. brigantiaca, P. mandshurica, P. mume, P. dasycarpa,...*
	***Amygdalus* (L.) Benth Hook.**	***Euamygdalus***	*P. davidiana, P. dulcis, P. ferganensis, P. kansuensis, P. mira, P. persica,…*
		Chameamygdalus	*P. tenella*
	***Cerasus* (Adans.) Focke**	***Eucerasus***	*P. avium, P. cerasus, P. fruticosa,…*
		Microcerasus	*P. besseyi, P. tomentosa,…*
		Pseudocerasus	*P. serrula, P. serrulata, P. canescens,…*
		Phyllocerasus	*P. pilosiuscula*
		Mahaleb	*P. mahaleb,…*
		Phyllomahaleb	*P. maximowiczii*
		Lobopetalum	*P. dielsiana,…*
	***Padus* (Moench) Focke**		*P. padus, P. virginiana,…*
	***Laurocerasus* (Ser.) Rehd.**		*P. lusitanica* , *P. laurocerasus*

1-2. ESPECIES FRUTALES DE HUESO DEL GÉNERO *Prunus.* EL MELOCOTONERO

Dentro del género *Prunus*, subgénero *Amygdalus*, existen especies frutales de gran importancia socio-económica, como el melocotonero (*P. persica*) y el almendro (*P. dulcis*). Estas dos especies representan la mayor superficie cultivada de frutales de hueso en España, con una superficie de 611.920 ha para el almendro y de 89.454 ha para el melocotonero (Figura 1-1). El cerezo (*P. avium*), perteneciente al subgénero *Cerasus*, ocupa una menor superficie (49.112 ha), comparada con el melocotonero y el almendro, seguido por el ciruelo (19.236 ha) y el albaricoquero (19.098 ha). En cuanto a la producción anual en España, la mayor producción corresponde al melocotonero (1.130.800 t), seguida de la de ciruelo, almendro, albaricoquero y, por último, cerezo (Figura 1-1).

El melocotonero [*Prunus persica* (L.) Batsch] es una especie que pertenece a la familia *Rosaceae,* subfamilia *Prunoïdeae,* género *Prunus*, subgénero *Amygdalus* (L.) Focke, sección *Euamygdalus* Schneid. Es uno de los frutales más tecnificado y más difundido en todo el mundo. España es la segunda productora a nivel europeo, con más de un millón de toneladas, y la cuarta a nivel mundial. El 70% de la producción se destina al consumo en fresco, generalmente para mercado interior; en torno al 20% a su procesado industrial, y sólo el 10% se destina a la exportación. Se trata de un frutal de zona templada no muy tolerante al frío, y su área de cultivo se extiende entre los 30 y 45º de latitud norte y sur.

A principios del siglo XX, algunos programas de mejora seleccionaron, a partir de poblaciones procedentes de semilla, genotipos de melocotonero por su buena productividad y comportamiento (Hesse, 1975; Scorza y Okie, 1990) que se propagaron clonalmente por medio del injerto. Además, algunas variedades procedentes de China, de frutos grandes y con buena calidad, se introdujeron en los programas de mejora norteamericanos y se utilizaron extensivamente como parentales para la obtención de nuevas variedades de melocotonero. Las variedades tradicionales, cultivadas hasta entonces, fueron reemplazadas por las nuevas selecciones procedentes de dichos los programas de mejora, reduciéndose drásticamente la diversidad del melocotonero en USA (Scorza et al., 1985; Werner y Okie, 1998).

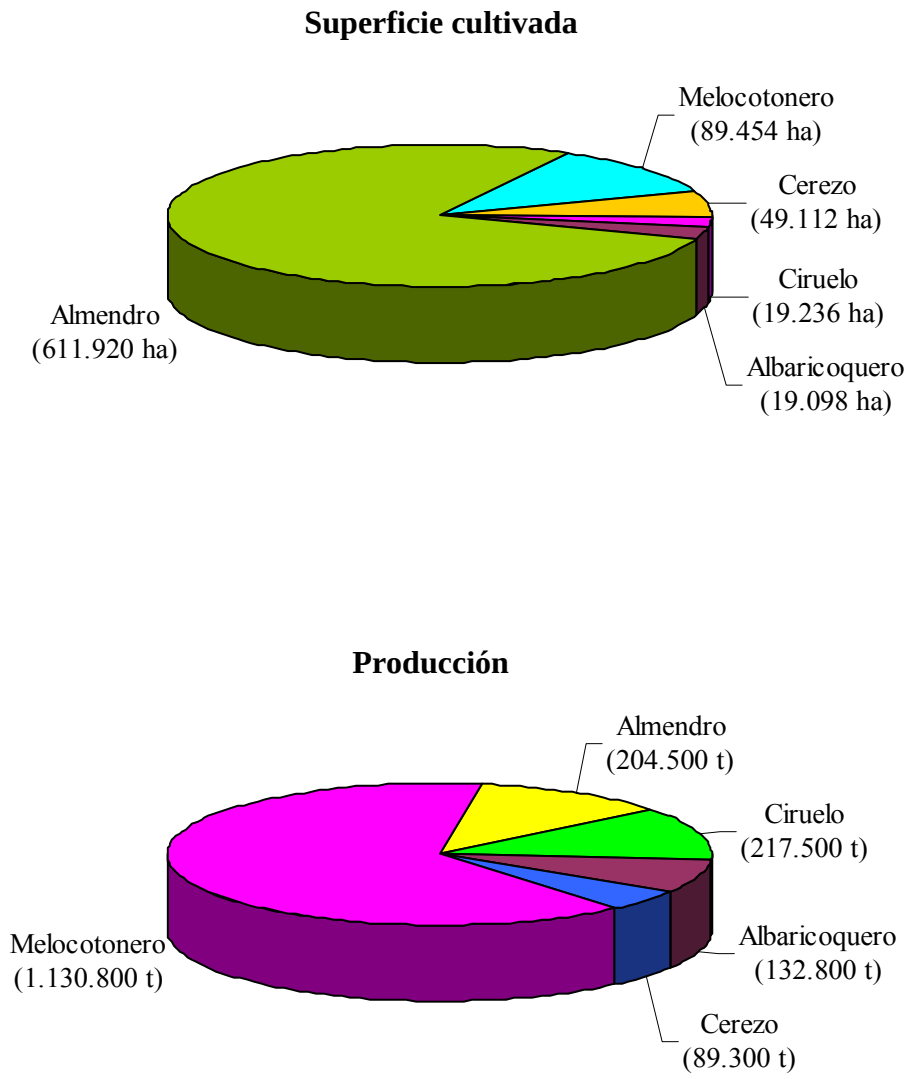

***Figura 1-1**. Superficie cultivada y producción en 2005 de los principales cultivos de frutales de hueso en España (FAOSTAT, 2006).*

Los descendientes de esas variedades chinas y sus cruzamientos fueron introduciéndose también en Europa, sustituyendo a los cultivares locales, lo que llevó a la pérdida de la biodiversidad que existía en el continente europeo (Scorza et al., 1985; Badenes et al., 1998).

El melocotonero es la especie frutal con un mayor dinamismo varietal ya que cada año aparecen numerosas novedades en el mercado, y la renovación varietal es una de las más intensas. Debido a las características climáticas y de producción, la distribución varietal no sólo varía con la fecha de maduración sino también con las zonas de cultivo.

La mayoría de los programas de mejora van dirigidos a conseguir características específicas, mejorando la calidad del fruto en cuanto al aspecto y las características organolépticas. Entre otras, la firmeza del fruto es una característica de gran importancia, ya que condiciona su manejo y transporte. Esta característica divide las variedades en dos tipos: de carne blanda ("melting") y de carne dura ("non-melting"). Los frutos de carne dura tienen una mejor aptitud para el manejo y el transporte, y suelen utilizarse en la industria conservera, aunque en España también tienen valor comercial para su consumo en fresco.

Los principales criterios de selección empleados en las variedades de melocotonero son: requerimientos edafoclimáticos (necesidades en horas frío), destino del fruto (consumo en fresco o procesado industrial), demanda del mercado, época de producción o maduración, área de producción y calidad del fruto.

1-3. PATRONES PARA FRUTALES DE HUESO DEL GÉNERO *Prunus*

Un árbol frutal está generalmente compuesto por dos partes genéticamente diferentes, que pueden pertenecer a la misma o distinta especie: la parte aérea o variedad y la raíz o patrón (Felipe, 1989).

El uso de patrones frutales permite la adaptación de la variedad a condiciones edáficas que, en algunos casos, son desfavorables para la especie, además de conferir resistencia y/o tolerancia a enfermedades y patógenos del suelo (Garner, 2003). Además, el patrón es el principal responsable de la nutrición mineral de la planta.

La importancia del patrón sobre el comportamiento vegetativo y productivo de la variedad injertada es bien conocido (Guerriero et al., 1984; Byrne, 1988). Entre los principales factores en los que influyen los patrones sobre la variedad cabe destacar: el vigor o desarrollo del árbol (Wertheim y Webster, 2005); la fecha de floración y

productividad (Tabuenca y Gracia, 1971; Durner y Goffreda, 1992); la rapidez de entrada en fructificación, época de maduración y calidad de los frutos (tamaño, color, firmeza, composición mineral, contenido en azúcares y en ácidos orgánicos, etc.) (Caruso et al., 1996; Castle, 1995; Jiménez et al., 2004); la composición mineral en flores y hojas (Sanz y Montañés, 1995, Betrán et al., 1997; García et al., 2005); la sensibilidad a determinados factores bióticos limitantes en determinados suelos (plagas de insectos, nematodos, virus, hongos, y bacterias) (Howell, 1987; Pinochet et al., 1999; Lang y Howell, 2001; Thomidis et al., 2005). Existen además otros factores abióticos que afectan a la adaptación de las especies frutales, como la clorosis en suelos calizos y pesados (Moreno et al., 2001; Giorgi et al., 2005), asfixia o sequía (Okie, 1987; Moreno et al., 2001), temperaturas extremas (Lang et al., 1997) y suelos pobres en nutrientes (De Salvador et al., 2005).

Existen dos tipos de patrones para frutales de hueso que pueden clasificarse en patrones francos y patrones clonales (Agustí, 2004). Los patrones francos provienen de semillas de la misma especie que la variedad injertada (Howard, 1987), y se usan para algunos frutales de hueso. Sin embargo, en la actualidad, la propagación de los patrones frutales se hace de forma clonal (vegetativa), lo que asegura una uniformidad en el desarrollo vegetativo y en la producción del material utilizado (Felipe, 1989). Los patrones francos se mantienen en zonas donde no existe un patrón clonal adecuado.

Entre los patrones más utilizados actualmente para melocotonero se encuentran los híbridos almendro x melocotonero (*P. dulcis* x *P. persica*: GF 677, GF 557, GxN, Adafuel, etc.) (Cambra, 1990; Moreno y Cambra, 1994; Felipe, 1997) y los híbridos ínterespecíficos de melocotonero (*P. persica* x *P. davidiana:* Cadaman, Barrier, etc.), (Edin y Garcin, 1994). En cuanto al cerezo, los patrones más utilizados están representados por las especies *P. avium*, *P. mahaleb* (Santa Lucia) y *P. cerasus*, y son utilizados tanto para cerezo (*P. avium*) como para guindo (*P. cerasus*). Los patrones de ciruelo pertenecen, en general, a las especies *P. insititia* (ciruelo San Julián, Pollizo de Murcia, etc.), *P. domestica, P. cerasifera* (Mirobolán) y *P. cerasifera* x *P. munsoniana* (Mariana).

1-4. INTERÉS DE LA IDENTIFICACIÓN VARIETAL EN ESPECIES FRUTALES Y PATRONES DE *Prunus*

Los programas de selección y mejora de variedades y patrones frutales, deben ir acompañados de una correcta caracterización del material vegetal. La caracterización morfológica y fenológica de dichos patrones y variedades es imprescindible para que éstos puedan figurar como nuevas obtenciones vegetales en el registro de variedades protegidas de la Oficina Española de Variedades Vegetales (OEVV) del Instituto Nacional de Semillas y Plantas de Vivero (INSPV) y de la Oficina Europea (*Community Plant Variety Office*, CPVO). Por otro lado, la existencia de un número elevado de variedades crea la necesidad de encontrar métodos de caracterización e identificación más rápidos y eficaces, para facilitar el control de identidad del material propagado, tanto en una colección de plantas madres como en el proceso de su comercialización por las empresas viveristas al agricultor. Además, la identificación facilita la gestión de las colecciones de germoplasma, al determinar la presencia de homonimias y sinonimias, y proteger los derechos de los mejoradores, al comparar las nuevas variedades con las ya existentes. Finalmente, la caracterización permite detectar relaciones genéticas y filogenéticas entre especies y variedades, dando una información de gran utilidad en los programas de mejora genética (para el diseño de los cruzamientos), y en estudios filogeográficos.

1-5. CARACTERIZACIÓN E IDENTIFICACIÓN VARIETAL DE ESPECIES FRUTALES Y PATRONES *Prunus*

1-5-1. Caracterización morfológica

Tradicionalmente las distintas variedades de frutales se han identificado por sus características fenotípicas y, generalmente, con la priorización de aquellas que interesan más desde el punto de vista agronómico y las que la experiencia demuestra que son más estables. Dichas características hacen referencia a aspectos del: porte y vigor; morfología de ramas, flores, hojas, frutos y semillas; época de floración y maduración; calidad del fruto; resistencia/sensibilidad a plagas o enfermedades, etc. Sin embargo, los pomólogos no han utilizado idénticos criterios a la hora de valorar los diferentes caracteres, y por ello, la comparación de resultados ha dado lugar a muchas confusiones.

La Unión para la Protección de las Obtenciones Vegetales (UPOV), es un organismo internacional que tiene por objeto poner a punto la protección de las novedades varietales, y ha establecido modelos descriptivos para las distintas especies de frutales, determinando las directrices para la conducción y examen de caracteres distintivos de la homogeneidad y estabilidad.

Hasta ahora, los caracteres morfológicos han sido los únicos reconocidos por el Reglamento Técnico de Control y Certificación de plantas de vivero de frutales (Royo et al., 1994). Sin embargo, su exclusiva utilización presenta una serie de limitaciones que se refieren a continuación.

- Exigencia de disponer de un campo de variedades de referencia bastante amplio, a fin de poder comparar en condiciones ambientales similares.
- Exigencia de observar necesariamente flores y frutos, lo cual retrasa el proceso de identificación debido al periodo de juvenilidad de la especie y, por tanto, el proceso de registro.
- Los caracteres fenotípicos en los que se basa son, a veces, excesivamente dependientes del medio o de las circunstancias en las que se desarrolla la planta.
- En algunas ocasiones, diferencias agronómicas de gran interés no se corresponden con diferencias fenotípicas suficientes como para poder distinguir el material con total seguridad.

1-5-2. Caracterización molecular

Para evitar los problemas descritos anteriormente en la identificación en especies leñosas , los investigadores han buscado otros métodos alternativos a los morfológicos, más independientes del medio, y de análisis rápido en plantas jóvenes. La caracterización molecular con el desarrollo de los marcadores bioquímicos y moleculares ha permitido solventar estos problemas.

El descubrimiento y la utilización de los marcadores isoenzimáticos a finales de los años 70, marcaron una revolución en este sentido. Con los isoenzimas, se amplió el conocimiento de la estructura y heterogeneidad genética entre diferentes especies, variedades, y poblaciones de distinto origen geográfico. En la genética frutal han sido utilizados ampliamente, con resultados significativos en diversos aspectos de mejora (Messeguer et al., 1987). Estos marcadores tienen la ventaja de caracterizar el genotipo de un individuo a partir de muestras de células o de tejidos.

Los avances de la tecnología del ADN recombinante han permitido el desarrollo de los marcadores moleculares basados en el ADN, consiguiendo estabilidad en la identificación de especies y variedades. Inicialmente, la utilización de enzimas de restricción del ADN (RFLP- *Restriction Fragment Length Polymorphism*) (Grodzicker et al., 1974) y posteriormente el desarrollo del proceso de amplificación en cadena mediante reacción en cadena de la polimerasa (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988) llevó a la descripción de otras clases de marcadores moleculares. El uso intensivo de esta nueva tecnología contribuyó al descubrimiento y al estudio de diversas clases de secuencias repetitivas de ADN, llamadas mini y microsatélites, otra fuente rica en polimorfismo genético. Actualmente, se puede obtener un número ilimitado de marcadores moleculares altamente polimórficos, en cualquier organismo vivo a través de diversas técnicas.

1-5-2-1. Tipos de marcadores moleculares

1-5-2-1-1. Los marcadores isoenzimáticos

El principio básico de la técnica reside en el uso de electroforesis en gel de almidón (Smithies, 1955) y en la visualización del producto enzimático por métodos histoquímicos (Hunter y Market, 1957). En mejora de plantas, las isoenzimas han sido utilizadas para la detección de ligamiento genético con caracteres mono y poligenéticos, identificación de variedades, selección indirecta de caracteres agronómicos, introgresión génica y evaluación de germoplasma (Tanksley and Orton, 1983). Los marcadores isoenzimáticos se aplicaron en especies leñosas para la identificación de melocotonero (Arulsekar et al., 1986), almendro (Arulsekar et al., 1986; Cerezo et al., 1989), albaricoquero (Battistini y Sansavini, 1991; Manganaris et al., 1999), vid (Royo et al., 1997) y cítricos (Leitao et al., 2000). La detección de las isoenzimas incluye básicamente tres pasos: 1) extracción de proteínas del tejido vegetal, 2) separación de estas proteínas a través de electroforesis y 3) coloración histoquímica del gel, lo que permite la visualización del producto de la reacción enzimática en forma de una banda.

Los alelos isoenzimáticos son codominantes, es decir, los genotipos heterocigotos y homocigotos de un determinado *locus* son fácilmente identificados. Esto permite estimar directamente parámetros tales como frecuencias genotípicas, frecuencias alélicas así como coeficientes de diversidad genética y heterocigosidad (Weir, 1990).

Sin embargo, las isoenzimas presentan tres limitaciones básicas: 1) el número total de *loci* que pueden ser detectados en el genoma es limitado, 2) el número de alelos por *locus*, es decir, el nivel de polimorfismo genético detectable en cada *locus* es generalmente bajo, entre 10 y 20 por especie (Murphy et al., 1990), y 3) varía la expresión en tejidos. Este nivel de resolución no permite la cobertura completa del genoma y por consiguiente, limita ciertas áreas de estudio como, por ejemplo, la construcción de mapas genéticos saturados.

1-5-2-1-2. RFLPs (*Restriction Fragment Length Polimorphism* / *Polimorfismo en el tamaño de los fragmentos de restricción)*

El análisis del polimorfismo de longitud de fragmentos de restricción (RFLP) fue una de las primeras técnicas que se utilizó para detectar variaciones a nivel de la secuencia del ADN. Esta tecnología se desarrolló en los años 70 (Grodzicker, 1974) y se aplicó inicialmente en el estudio del genoma humano (Botstein et al., 1980). El principio de la técnica RFLP se basa en la detección de fragmentos de DNA de distinto peso molecular, a partir de moléculas de DNA, de diferentes individuos, que han sido sometidas a digestión con enzimas de restricción. Las diversas mutaciones que afectan a las moléculas de DNA modifican los lugares de restricción y producen fragmentos de longitud variable. Estas diferencias de longitud de los fragmentos, se observan por electroforesis en geles de agarosa. El patrón de bandas es tan complejo que es necesario utilizar sondas específicas para visualizar sólo algunos fragmentos que se transfieren a membranas mediante la técnica de Southern blot. Las sondas de DNA que hibridan en zonas específicas suelen corresponder a genes conocidos. Los RFLPs han sido una herramienta muy útil en el mapeo genético de frutales como en manzano (Hemmat et al., 1994), en melocotonero (Rajapakse et al., 1995; Dirlewanger et al., 1998) y para el estudio de la diversidad genética en las colecciones de germoplasma en vid (Gogorcena et al., 1993), albaricoquero (De Vicente et al., 1998), y melocotonero (Quarta et al., 2000).

La ventaja de los RFLPs frente a las isoenzimas, es que pueden cubrir todo el genoma del organismo estudiado. El uso de RFLP aumenta, por lo tanto, la probabilidad de encontrar asociaciones estadísticamente significativas entre estos marcadores y genes que controlan un carácter de interés y además, son codominantes. El número de marcadores RFLP es prácticamente ilimitado, y el nivel de polimorfismo alélico en cada

locus es muy superior al obtenido con los izoenzimas. Los RFLPs presentan entre sus ventajas la repetibilidad de los resultados, permitiendo la comparación de trabajos. Los RFLPs una vez situados en el mapa genético de una población pueden ser utilizados para estudios de ligamientos en otras poblaciones. Sin embargo, la técnica RFLP presenta ciertos inconvenientes, como su lentitud y complejidad. Además, su elevado coste limita su uso en un número elevado de individuos. Estos inconvenientes hacen que el uso de esta técnica haya diminuido en los últimos años, y con la llegada de l*a* técnica de la PCR (Reacción en Cadena de la Polimerasa o *Polymerase Chain Reaction*) se han desarrollado nuevos marcadores moleculares que han sustituido casi totalmente el uso de los RFLPs.

1-5-2-1-3. Marcadores basados en la reacción PCR

La tecnología de la reacción en cadena de la polimerasa (PCR) fue concebida a mediados de la década de los 80 (Saiki et al., 1985; Mullis and Faloona, 1987) y desde entonces, causó una verdadera revolución en la biología molecular. La facilidad, la rapidez, la versatilidad y la sensibilidad de la PCR, hace que sea una técnica poderosa para estudios genéticos y moleculares que incluyen gran número de individuos de cualquier organismo vivo. La PCR consiste en la síntesis enzimática *in vitro* de millones de copias de un segmento específico de ADN, en presencia de la enzima polimerasa. La reacción de PCR se basa en el apareamiento y la polimerización enzimática de un par de oligonucleótidos utilizados como cebadores (*primers*) que delimitan una secuencia determinada de ADN de doble cadena. Los cebadores son sintetizados artificialmente, de manera que sus secuencias de nucleótidos sean complementarias a las secuencias específicas que flanquean la región del ADN que deseamos amplificar. Un ciclo de PCR está compuesto de tres etapas: desnaturalización, hibridación y extensión (Figura 1-2). En primer lugar, se desnaturaliza el ADN (se separan las dos hebras de las cuales está constituido). Este paso puede realizarse de diferentes modos, siendo el calentamiento (95ºC) de la muestra la más habitual. A continuación, se producirá la hibridación, es decir, el cebador se unirá a su secuencia complementaria en el ADN molde. Para esto es necesario que la temperatura descienda (generalmente a 55ºC, aunque se puede variar según sea el caso). Por último, se aumenta la temperatura hasta 72ºC, temperatura a la cual la polimerasa presenta su máximo de actividad, produciéndose una copia del fragmento que se desea amplificar mediante la adición de nucleótidos. Este ciclo

(desnaturalización-hibridación-extensión) se repetirá de acuerdo a los fragmentos amplificados que se desee. Generalmente son 30 ciclos, ya que un número mucho mayor no implica un mayor rendimiento. Esta escala de amplificación permite, por lo tanto, iniciar la PCR con cantidades mínimas de ADN (del orden de picogramos o nanogramos) y terminar la reacción con grandes cantidades de ADN de la secuencia específica de interés.

1-5-2-1-3-1. RAPDs (Random Amplified Polymorphic DNA / DNA polimórfico amplificado al azar)

Los marcadores del tipo RAPDs fueron desarrollados por Williams et al. (1990), y fueron los primeros marcadores basados en la PCR. Los RAPDs son secuencias de ADN del genoma, amplificadas al azar, utilizando cebadores cortos y con unas condiciones de PCR que favorecen la amplificación inespecífica de varias bandas. Esta técnica detecta polimorfismo entre individuos cuando uno o los dos cebadores no se hibrida porque existen diferencias en la secuencia complementaria a los cebadores (Cushwa y Medrano, 1996). Las inserciones, deleciones o inversiones en el ADN también generan polimorfismo por presencia/ausencia de bandas o variaciones en el tamaño de éstas (Williams et al., 1990). La facilidad de realización, mediante amplificación por PCR y análisis por electroforesis en gel de agarosa, la convierten en una técnica fácil, rápida y de bajo coste.

Son muchos los trabajos en los que se ha utilizado este tipo de marcadores, y con aplicaciones muy diversas, como su uso en análisis de variabilidad e identificación varietal de diversas especies (Gogorcena et al., 1994; Howell et al., 1994; Warburton y Bliss, 1996; Badenes et al., 1998; Quarta et al., 2000), y en construcción de mapas de ligamiento en *Prunus* (Chaparro et al., 1994; Rajapakse et al., 1995; Dirlewanger et al., 1996). Los RAPDs se han usado también para identificar marcadores ligados a caracteres de interés, como los que determinan el color de la carne, la adherencia de ésta al hueso y la textura del fruto en melocotonero (Warburton et al., 1996).

Los RAPDs, a diferencia de las isoenzimas y los RFLPs, son marcadores dominantes en su mayoría, es decir, no se puede distinguir entre individuos homocigotos y heterocigotos. Sólo se detecta un alelo, mientras que las demás variaciones alélicas son clasificadas conjuntamente como un alelo nulo. El bajo contenido de la información genética por *locus* constituye la principal desventaja de estos marcadores moleculares,

junto con los problemas de baja repetibilidad en los análisis (Benter et al., 1995; Cushwa y Medrano, 1996).

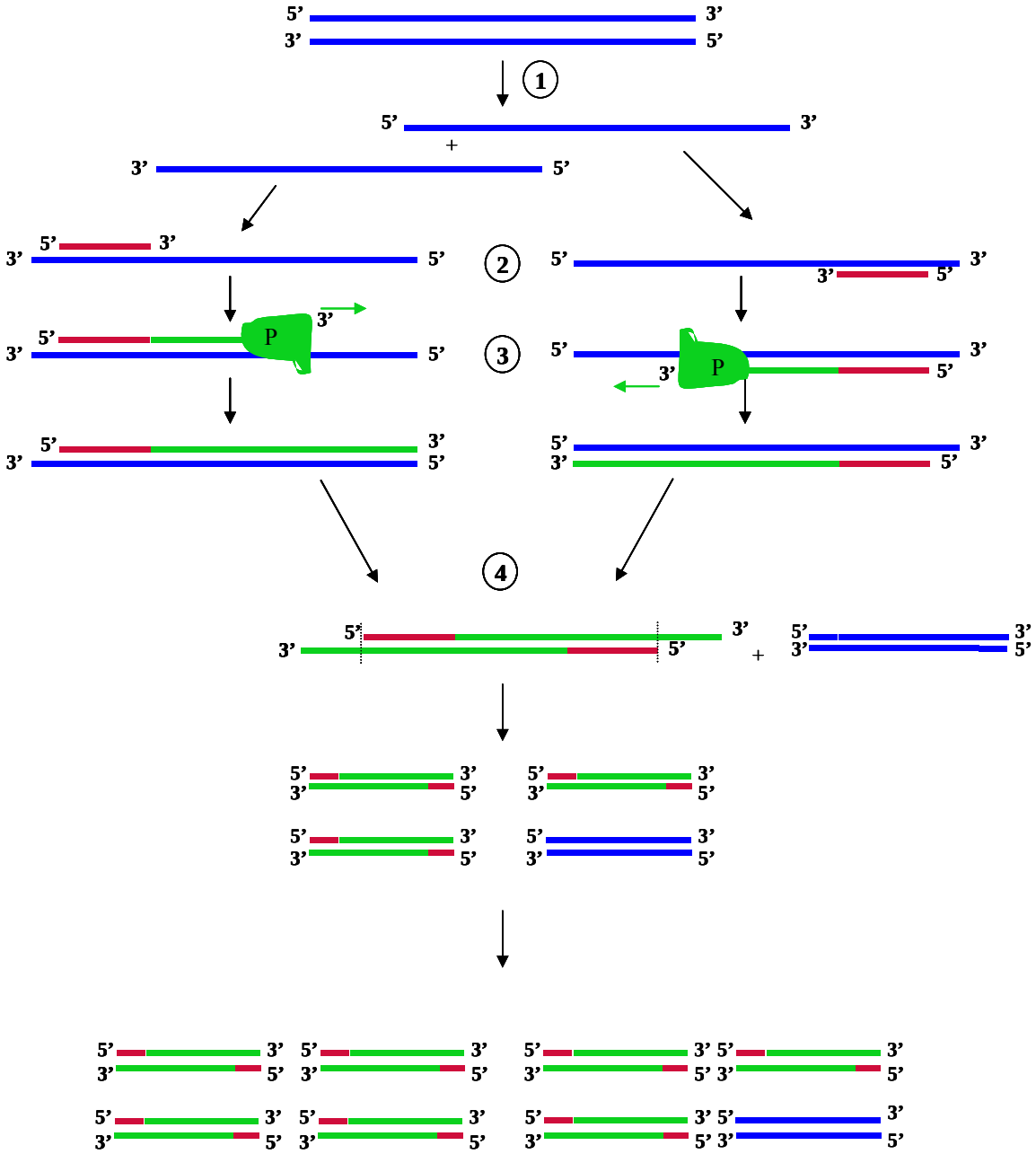

Figura 1-2. *Etapas de un ciclo de PCR (polymerase chain reaction)*

1- Desnaturalización a 95°C.

2- Hibridación a 55°C.

3- Extensión a 72°C (P=Polimerasa).

4- El primer ciclo está completo. Las dos hebras de ADN obtenidas sirven para el próximo ciclo para duplicar la cantidad de ADN.

1-5-2-1-3-2. AFLPs (Amplified Fragment Length Polymorphism / Polimorfismo de la longitud de los fragmentos amplificados)

La técnica AFLP fue desarrollada por Vos et al. (1995). Esta técnica se basa en la amplificación selectiva de fragmentos de DNA obtenidos tras la digestión con enzimas de restricción. Este método combina por lo tanto, la especificidad proporcionada por la digestión con enzimas, que reconocen secuencias específicas del genoma, con la rapidez y facilidad de detección de polimorfismos mediante la amplificación por PCR. Modificando los nucleótidos de selección en la zona de restricción, se amplifican grupos de fragmentos específicos, por lo que el empleo de la técnica AFLP permite la identificación de un número ilimitado de marcadores.

La aplicación de AFLPs en vid, ha permitido distinguir clones y variedades, por lo que es una herramienta muy útil para la selección clonal (Ribaut et al., 1997; Cervera et al., 1998; Scott et al., 2000). Se ha utilizado además para la elaboración de mapas genéticos en melocotonero (Dirlewanger et al., 1998; Lu et al., 1998; Wang et al., 2000) y para la obtención de marcadores asociados a caracteres de interés, como la resistencia a nematodos en melocotonero (Lu et al., 1998). Numerosos estudios genéticos, filogenéticos y de caracterización se han basado en AFLPs, como es el caso de estudios en germoplasma de vid y de melocotonero (Fossati et al., 2001; Blenda et al., 2005) y estudios de relaciones genéticas en albaricoquero japonés (*Prunus mume* Sieb. et Zucc.) (Fang et al., 2006).

La ventaja más destacada de la técnica es el gran número de marcadores analizados de forma simultánea en un único gel, y por tanto con un gran poder de detección de variabilidad genética. La principal limitación de los marcadores AFLPs es el bajo contenido de información genética por *locus*. Estos marcadores son dominantes y los datos tienen naturaleza binaria (presencia/ausencia de un alelo), lo que limita su utilización en campos donde se necesita identificar los *loci* a los que pertenecen las bandas.

1-5-2-1-3-3. Microsatélites o SSRs (Sequence Simple Repeat / Secuencias simples repetidas)

Los microsatélites son unas regiones del genoma de animales y plantas que consisten en una serie de repeticiones de secuencias cortas (motivos) de nucleótidos, por ejemplo,

$(CAC)_n$, $(GACA)_n$, $(TA)_n$, $(GT)_n$, $(GATA)_n$, etc. Estas regiones no son codificantes y su origen y su función no están claros. Presentan la particularidad de que el número de repeticiones de los motivos básicos que las constituyen es muy variable, y puede diferir de un individuo a otro (Morgante y Olivieri, 1993). Por tanto, analizando estas regiones es posible identificar individuos en poblaciones animales o vegetales. La forma más sencilla de analizar la variación de las regiones de microsatélites es mediante la reacción de PCR. Se sintetizan cebadores que son complementarios a las regiones flanqueantes del microsatélite, y las diferencias en el número de repeticiones del motivo básico del microsatélite se amplificarán y visualizarán como fragmentos de ADN de diferente longitud (Figura 1-3). Cada una de las regiones microsatélites constituye un *locus* genético y los diferentes tamaños de bandas que se pueden amplificar constituyen los diversos alelos de ese *locus* (Ferreira y Grattapaglia, 1998).

Los SSR son muy frecuentes en el genoma y están distribuidos al azar, permitiendo la más completa cobertura de cualquier genoma eucariota. Teniendo en cuenta la expresión codominante y el multialelismo, los marcadores SSR son los que poseen el más elevado contenido de información de polimorfismo, o PIC (*Polymorphism Information Content*) en la terminología de marcadores moleculares. La técnica es muy adecuada para estudios de paternidad, tanto en aplicaciones forenses humanas como en mejora genética animal y vegetal, dado que los dos alelos que aparecen en un individuo deben aparecer también en los dos presuntos parentales (Ferreira y Grattapaglia, 1998). La reproducibilidad de los resultados entre laboratorios ha llevado, en el caso de la vid, al desarrollo de bases de datos de referencia para todas las variedades (Sefc et al., 2000). Esta comparación de datos se ve facilitada por el hecho de que los resultados de microsatélites se expresan numéricamente, mediante el tamaño en pares de nucleótidos de los dos alelos de cada *locus*. La complejidad técnica de los microsatélites es de nivel medio. Dado que algunos alelos difieren únicamente en un nucleótido, hay que recurrir a una separación electroforética de los mismos en gel de acrilamida y tinción mediante nitrato de plata o mediante el secuenciador automático. El mayor inconveniente de los microsatélites es que no están disponibles para todas las especies cultivadas. Requieren un proceso laborioso de clonaje y secuenciación para determinar las secuencias flanqueantes de las regiones microsatélites para cada especie, con el fin de diseñar los cebadores adecuados. Muchos SSRs se han desarrollado en melocotonero y han dado amplificaciones también en otras especies *Prunus* (Downey e Iezzoni, 2000; Serrano et al., 2002; Romero et al., 2003; Zhebentyayeva et al., 2003). Los marcadores

desarrollados en melocotonero se han empleado para el estudio genético de esta especie, así como en otras especies del género *Prunus*, principalmente para el análisis de la diversidad genética, la identificación varietal y la construcción de mapas de ligamiento, localizando caracteres de interés (Wünsch y Hormaza, 2002; Aranzana et al., 2003; Vilanova et al., 2003).

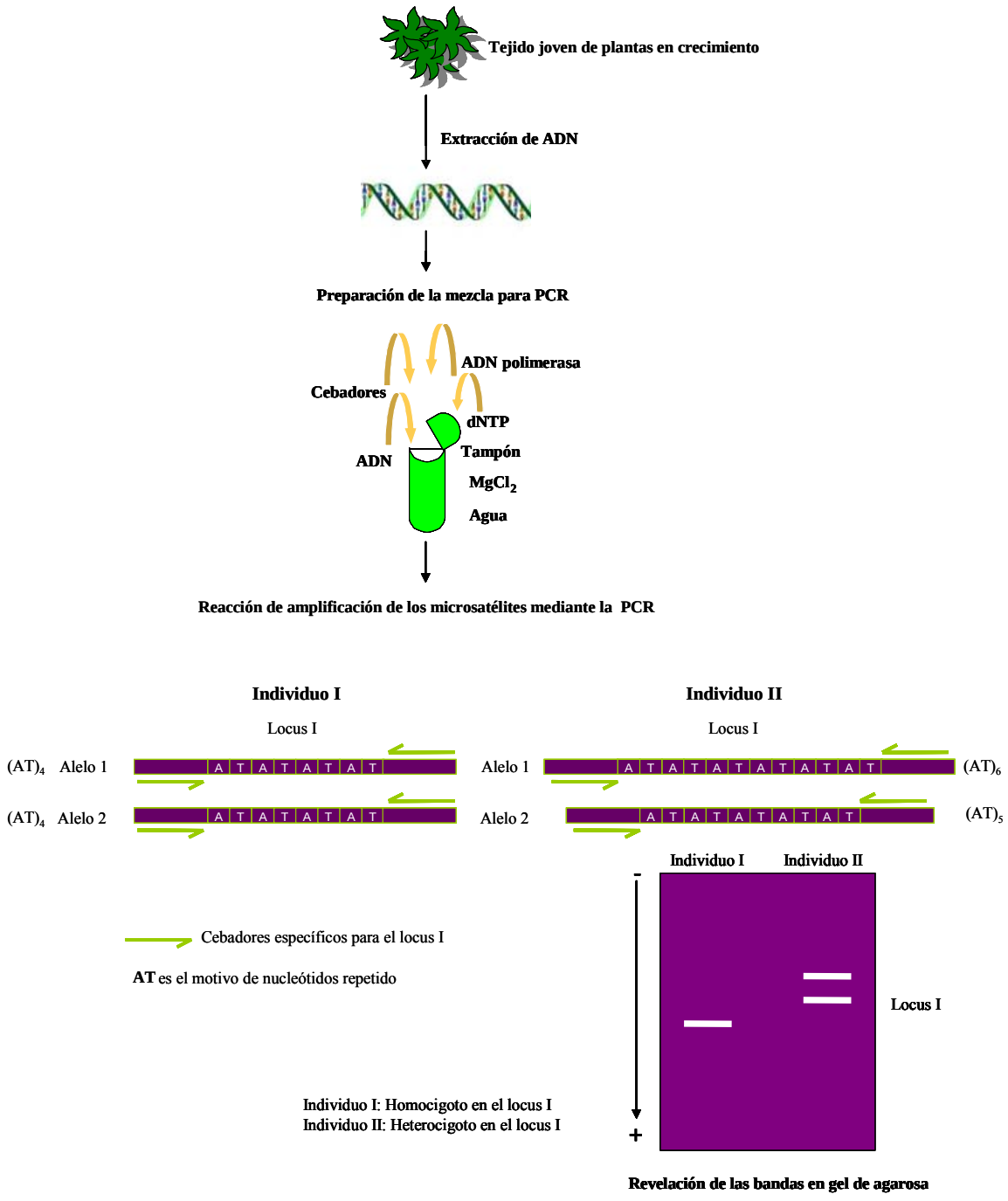

Figura 1-3. *Esquema del procedimiento de amplificación de los microsatélites en plantas y revelación del polimorfismo en SSRs mediante la PCR.*

1-5-2-1-3-4. PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism / Reacción en cadena de la polimerasa – Polimorfismo en el tamaño de los fragmentos de restricción)

Estos marcadores son codominantes y están generados por la combinación de amplificaciones mediante la técnica de PCR y digestiones con enzimas de restricción. Existen varios métodos para detectar polimorfismo después de una amplificación de PCR. Uno de ellos, consiste en digerir los fragmentos amplificados con diversos enzimas de restricción hasta encontrar un enzima que produzca un polimorfismo de digestión. El polimorfismo así obtenido se conoce como PCR-RFLP o CAPS (*Cleaved Amplified Polymorphic Sequence*) (Konieczny y Ausbel, 1993). Los productos amplificados y digeridos con enzimas de restricción, son posteriormente separados en geles de agarosa y visualizados con bromuro de etidio (Figura 1-4).

Esta técnica (PCR-RFLP) se ha utilizado para estudios filogenéticos en *Prunus* (Badenes y Parfitt, 1995; Panda et al., 2003). Mohanty et al. (2001; 2002) utilizaron también este método para determinar la filogeografía y la diversidad entre poblaciones de ciruelo endrino (*P. spinosa* L.) y cerezo (*P. avium* L.).

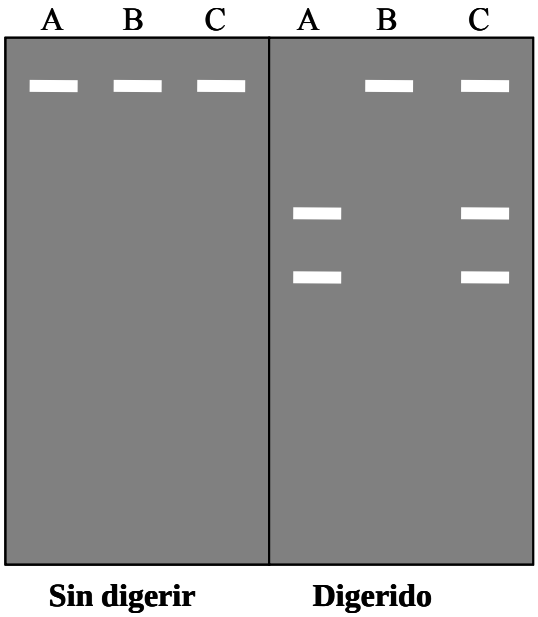

Figura 1-4. *Esquema representativo de los marcadores tipo PCR-RFLP o CAPS. Las muestras A, B y C a la izquierda producen bandas del mismo tamaño, éstas representan las amplificaciones de PCR en tres individuos, antes de digerir con el enzima de restricción. A la derecha, después de digerir, se observa un lugar de corte en la muestra A, ninguno en la muestra B, y la muestra C presenta los dos alelos por tratarse de un híbrido entre A y B.*

1-5-2-1-3-5. Las secuencias ESTs (Expressed Sequence Tags / Etiquetas de secuencias expresadas)

Los ESTs son los marcadores genéticos desarrollados más recientemente, son codominantes y presentan secuencias del DNA complementario. La mayoría de los ESTs son generadas a partir de la región del 3'UTR (3' *untranslated region of the mRNA*). Así pues, el DNA complementario o cDNA corresponde a la copia del RNA mensajero o mRNA que migra del núcleo celular conteniendo la información codificada y por traducción, dirigirá la síntesis de polipéptidos (Russel, 1992).

Las secuencias EST son fáciles de obtener y altamente transferibles entre especies cercanas (Liu et al., 1999; Scott, 2001). Para la amplificación de ESTs se requiere el diseño de primers. Con esta técnica también se pueden obtener microsatélites a bajo coste, alto rendimiento e identificación de genes útiles para aplicaciones particulares

(Liu, 2003). La desventaja de las EST es que el aislamiento del RNAm es más complicado. Por otro lado, los intrones que pueden contener información genética no expresada, no forman parte del cDNA.

Las aplicaciones de los EST se basan en el hecho de que se originan a partir de segmentos de secuencias génicas y permiten, por consiguiente, la comparación de la diversidad génica en diferentes organismos, el estudio de la evolución génica, la búsqueda de supuestos ortólogos en bases de datos, la concepción de sondas para estudios de expresión génica, y la detección de SNPs (*Single Nucleotide Polymorphisms*).

1-5-2-1-3-6. SNPs (Single Nucleotide Polymorphism / Polimorfismo en un solo nucleótido)

Los SNPs son el reflejo de los cambios que pueden presentarse en el material hereditario a lo largo de la historia evolutiva de una especie. Estos cambios pueden identificarse en las secuencias del DNA y proveen información acerca de la historia evolutiva de las poblaciones humanas (Wang et al., 1998). Un SNP se presenta cuando existe la sustitución de un solo nucleótido por otro en una región específica del material genético, que puede pertenecer a un gen, el cual codificará una cadena polipeptídica con características diferentes a la cadena original y, a su vez, presentará un posible cambio en el fenotipo (Vignal et al., 2002). Este tipo de variaciones puede formar parte de la región no codificante del gen, como podría ser un intrón o un espacio intergénico, pero cuya presencia puede asociarse con una característica específica. Cabe mencionar también que los SNPs tienen una gran importancia en la identificación y etiología de diversas enfermedades.

El material genético puede presentar cambios en sus constituyentes, los nucleótidos de adenina (A), guanina (G), citosina (C) y timina (T), presentes en una secuencia cualquiera, pueden variar constituyendo esencialmente una mutación. En la secuencia ATTCAGG**A**C puede presentarse la variante ATTCAGG**C**C, en la cual el nucleótido resaltado muestra ese cambio (Figura 1-5). Si al comparar varias muestras con la misma secuencia general, se encuentra que este cambio es frecuente, es posible utilizarlo como un marcador genético. Ésta es la forma más común en que se presentan los SNPs. La frecuencia de distribución de los SNPs varía en cada genoma. En maíz, por ejemplo, se ha detectado 1 SNP por cada 60-120 pb (Ching et al., 2002), mientras que en el genoma

humano, se ha detectado 1 SNP por cada 1000 pb (Sachidanandam et al., 2001). El desarrollo de los marcadores de tipo SNP es extremadamente caro porque se basa en la secuenciación extensiva del genoma. Además de la secuenciación, otros métodos de detección de SNPs incluyen otras técnicas, como la PCR alelo-específica (Newton et al., 1989). La utilización de estos marcadores en plantas está todavía limitado a algunas especies, como *Arabidopsis* (Drenkard et al., 2000), tomate (Fridman et al., 2000), maíz (Tenaillon et al., 2001), albaricoquero japonés (*Prunus. mume* Sieb. et Zucc.) (Fang et al., 2006). Los SNPs se detectaron por primera vez en especies forestales (Le Dantec et al., 2004), y fueron utilizados para el análisis de las relaciones filogenéticas en *Citrus* por Novelli et al. (2004).

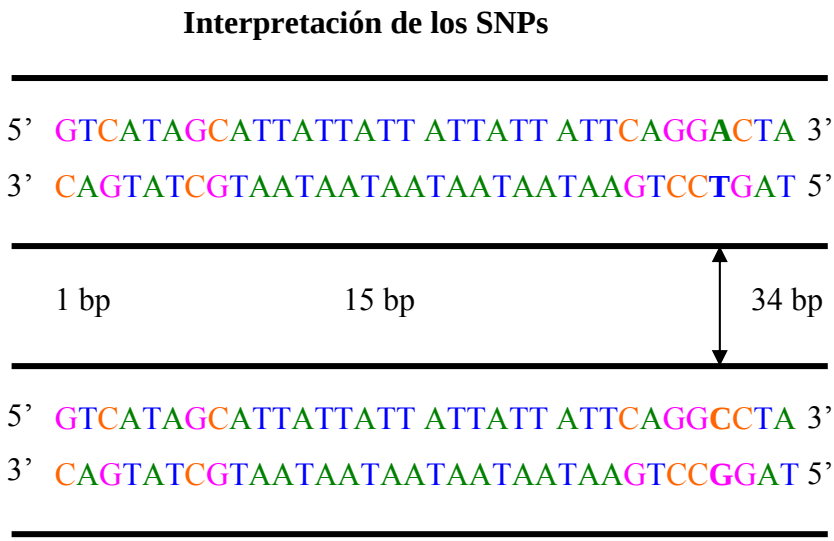

Figura 1-5. *Este dibujo esquemático del polimorfismo de un solo nucleótido muestra dos fragmentos de ADN (uno en la parte superior y otro en la inferior) que comparten la misma secuencia de 34 pares de bases y difieren sólo en el par de la posición 31 de los fragmentos. En esa posición, un A-T (parte superior) ha cambiado a un C-G (parte inferior).*

1-6. MÉTODOS DE ANÁLISIS DE LOS DATOS MOLECULARES

Los perfiles electroforéticos se pueden estudiar desde dos puntos de vista, cualitativo o cuantitativo. El estudio cualitativo (presencia o ausencia de bandas) se utiliza para determinar la similitud genética entre individuos. El estudio cuantitativo (intensidad de banda) permite revelar diferencias en actividad enzimática o en expresión génica. La similitud genética, obtenida tras la aplicación de técnicas de taxonomía numérica a los datos obtenidos (Crisci y López-Armengol, 1983; Romesburg, 1984), nos va a permitir conocer una estimación de la distancia genética entre los individuos bajo estudio. Los marcadores moleculares, tras la separación electroforética, son detectados como bandas. A partir de la información presentada por los perfiles electroforéticos, se obtienen unos datos binarios o de doble estado para cada carácter (banda) por genotipo, donde la presencia de una banda concreta se codifica como "1" y la ausencia de esta banda se codifica como "0". Este tipo de datos se obtiene, en general, con el uso de marcadores dominantes. En el caso del uso de marcadores codominantes, en especies diploides, los datos tomados de los perfiles electroforéticos son de triple estado, presencia de una banda (individuo homocigoto) y se codifica como "1", presencia de dos bandas (individuo heterocigoto) se codifica como "0,5", o ausencia de bandas se codifica como "0". A este último tipo de codificación de las bandas se denomina datos de frecuencia génica.

Con los datos obtenidos se construye una matriz básica de datos binarios (0/1) o matriz de frecuencias alélicas (0/0,5/1). Una matriz de distancias genéticas se obtiene a partir de la matriz básica, aplicando coeficientes de similitud que cuantifiquen la similitud genética.

Cuando disponemos de datos binarios de doble estado (presencia o ausencia), se calcula la similitud genética utilizando varios coeficientes de asociación, cuyos valores oscilan entre 1 (máxima similitud) y 0 (mínima similitud). Como ejemplos, se pueden citar:

- El coeficiente Simple "*Simple Matching Coefficient*" (Gower, 1985)
- El coeficiente de Jaccard (Jaccard, 1908)
- El coeficiente de Rogers y Tanimoto (1960)
- El coeficiente de Dice o Nei y Li (1979)

El coeficiente más adecuado para realizar estudios genéticos es el de Nei y Li (1979), por presentar ventajas sobre los otros (Lamboy et al., 1994). Este coeficiente tiene un significado biológico claro, es una estimación de la similitud entre dos muestras que son heredadas de un ancestro común. Además, es el coeficiente que presenta menos sesgo entre el valor real y el valor estimado cuando hay falsos positivos o falsos negativos en los datos.

Cuando se dispone de datos de triple estado (0/0,5/1), se calcula la similitud genética utilizando varios coeficientes de asociación, cuyos valores oscilan también entre 1 (máxima similitud) y 0 (mínima similitud). Se pueden citar:

- El coeficiente de Nei (1972)
- El coeficiente de Swofford-Olsens (1990)
- El coeficiente de Rogers (1972)
- El coeficiente de Prevosti (Wright, 1978)

El coeficiente más utilizado por los genetistas, en el caso de datos en frecuencias alélicas, es el coeficiente de Nei 1972 (Swofford y Olsen, 1990).

La matriz de similitud o de distancias genéticas expresa las relaciones entre la totalidad de los individuos estudiados, de par en par. Para observar las relaciones se necesita sintetizar la información de la matriz de similitud o de distancias genéticas, aplicando las técnicas del "análisis de agrupamiento" o del "método de ordenación". De esta forma se pueden observar gráficamente mediante un diagrama arborescente denominado "dendrograma", las relaciones genéticas entre los individuos con el grado de similitud entre sí.

Existen diferentes métodos de ordenación y se pueden citar como ejemplos:

Métodos ultramétricos: *UPGMA*

El método de reconstrucción filogenética UPGMA (*Unweight Pair Group Arithmetic Average*) se basa en las medias aritméticas y produce un árbol ultramétrico. A partir de la matriz de distancias entre los taxones en estudio, éstos se van a ir uniendo unos a otros por pares, comenzando por los que están a la mínima distancia (Sneath y Sokal, 1973).

Métodos aditivos: NJ (Neighbor Joining)

El método de *Neighbor-Joining* (NJ) fue desarrollado por Saitou y Nei (1987). Para aplicar este método, se parte de una matriz de distancias genéticas original que se transforma en una matriz de distancias modificadas, en la cual la separación entre cada par de taxones es reajustada en base a su divergencia media con todos los demás taxones. Se unen los dos taxones más próximos (mínima distancia), que forman un nudo, y se construye una nueva matriz de distancias con ese nudo sustituyendo a los dos taxones y reajustando nuevamente todos los valores. Se prosigue de forma sucesiva, creando sucesivos nudos, hasta incorporar todos los taxones.

Existen numerosos programas informáticos para evaluar la diversidad genética y representar árboles o dendrogramas. Como ejemplos: se pueden citar el programa PHYLIP (Felsenstein, 1993) aplicado en trabajos de análisis de diversidad en olivo (Angiolillo et al., 1999); el programa Arlequín (Schneider et al., 2000) utilizado para el análisis de variabilidad en especies de *Prunus* (Schueler et al., 2006), el programa MEGA (Kumar et al., 1993) utilizado por Aranzana et al. (2003) en estudios de variabilidad en melocotonero; GENEPOP (Raymond y Rousset, 1995) aplicado por Sefc et al. (2000) para cálculos de los parámetros de variabilidad en los microsatelites de vid. El programa NTSYS (Rohlf, 2000) es el programa más comúnmente utilizado por distintos grupos de trabajo en estudios de caracterización y diversidad genética en plantas (Mekuria et al., 1999; Lerceteau y Szmidt, 1999; Warburton et al., 2002; Aranzana et al., 2003), y es el programa que se utilizó para los análisis en esta tesis.

Para estudios de las relaciones filogenéticas entre organismos o especies se utiliza la cladística que es una rama de la biología que define las relaciones evolutivas entre los organismos basándose en similitudes derivadas. Es la más importante de las sistemáticas filogenéticas, que estudian las relaciones evolutivas entre los organismos. La cladística es un método de análisis riguroso que utiliza las "propiedades derivadas compartidas" de los organismos que se están estudiando. El análisis cladístico forma la base de la mayoría de los sistemas modernos de clasificación biológica, que buscan agrupar a los organismos por sus relaciones evolutivas. En contraste, la fenética agrupa los organismos basándose en su similitud global.

Como resultado final del análisis cladístico, se obtienen diagramas de relación en árbol llamados "cladogramas", para mostrar las distintas hipótesis sobre las relaciones. Un análisis cladístico puede basarse en tanta información como el investigador seleccione. La investigación sistemática moderna suele basarse en una gran variedad de información, incluyendo secuencias de ADN (datos moleculares), datos bioquímicos y datos morfológicos.

Para este tipo de análisis y representaciones de árboles genealógicos se utiliza principalmente el programa PAUP de Swofford (2002) que se aplicó en estudios filogenéticos en *Prunus* (Bortiri et al., 2001; Katayama y Uematsu, 2005).

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CAPÍTULO 2

Objetivos

El objetivo principal de esta tesis es evaluar la biodiversidad en frutales de hueso, en variedades de melocotonero y en patrones de *Prunus* de distintas colecciones. Basándose en la eficiencia de los marcadores moleculares, especialmente los microsatélites (SSRs) para la caracterización, y la técnica PCR-RFLP en estudios filogenéticos, se han planteado los siguientes objetivos específicos:

1- La caracterización molecular de la variedad de melocotonero "Miraflores" [*P. persica* (L.) Batsch] y de otras variedades tradicionales cultivadas en el Valle Medio del Ebro, así como la búsqueda de relaciones genéticas entre ellas, utilizando los marcadores microsatélites (SSRs) (Capítulo 3).

2- El análisis de la variabilidad y el estudio de las relaciones genéticas existentes entre las variedades de melocotonero del banco de germoplasma nacional de referencia, incluyendo germoplasma local español y algunas variedades comerciales de interés, utilizando los marcadores SSRs (Capítulo 4).

3- La caracterización molecular de patrones seleccionados o en fase de selección del programa de mejora de la Estación Experimental de Aula Dei, y el análisis de la diversidad genética entre patrones de distintas especies e híbridos inter-específicos (Capítulo 5).

4- La identificación de los parentales maternos en cruzamientos inter-específicos de *Prunus* y la búsqueda de relaciones genéticas y filogenéticas mediante la aplicación de la técnica PCR-RFLP (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism*) en el ADN cloroplástico (Capítulo 6).

CAPÍTULO 3

Molecular characterization of Miraflores peach variety and relatives using SSRs

ABSTRACT

Some traditional peach varieties, originated from the region of Aragón (Spain), were analysed by SSRs (Simple Sequence Repeats). The aim of this research was to characterize 19 clones related to 'Miraflores' variety, with unknown pedigrees, to assess their genetic diversity and to elucidate their possible relationships with 10 traditional peach varieties. Twenty SSR primer pairs with high levels of polymorphism, which have been previously developed for peach, were used in this study. A total of 46 alleles were obtained for all the microsatellites studied, ranging from one to six alleles per locus, with a mean value of 2.3 alleles per locus. Fourteen SSRs were polymorphic in the set of varieties studied and permitted to distinguish 16 different genotypes out of the 30 initially studied, although fourteen 'Miraflores' clones showed identical gel profiles. The genetic distance matrix was used to construct Neighbor joining cluster and to perform principal coordinate analysis which allowed the arrangement of all the genotypes according to their genetic relationships. The genetic relationships among these traditional peach varieties, and in particular among 'Miraflores' clones are discussed. The obtained results confirm that microsatellite markers are very useful for these purposes.

Keywords: Clones, Genetic diversity, Genetic relationships, Microsatellites, 'Miraflores' peach variety, Traditional peach varieties

3-1. INTRODUCTION

Peach [*Prunus persica* (L.) Batsch] is a member of the *Rosaceae*, and it is a diploid species with a basic chromosome number of x=8 and 2n=16. The *Prunus persica* is thought to be originated in China and spread to the rest of the world by means of seeds (Layne, 1987). It is a species well adapted to temperate and subtropical regions, between latitudes of 30º and 45º North and South (Westwood, 1978). The EU (European Union) is one of the main cultivation regions, with approximately 4.5 million metric tons annual production of peaches and nectarines (28% of the world production). The four countries: Italy, Spain, Greece, and France, ensure 92.7% of this production (FAOSTAT, 2005), Italy being the greatest peach producer of them (1,750,000 metric tons) followed by Spain (1,130,800 metric tons). Peach is one of the most economically and socially important deciduous fruit tree species. Therefore, precise cultivar identification and characterisation is essential for improving and securing peach culture in the world.

The official methods used to characterize and identify varieties in fruit tree species are based on morphological characterization and phenological observations according to UPOV (Unité pour la Protection des Obtentions Végétales) and IPGRI (International Plant Genetic Resources Institute) descriptors. This approach requires time, and the morphological characters can be subject to environmental influences. New molecular methods have been incorporated to characterize the varieties at the DNA level (see for a review: Martínez-Gómez et al., 2003). Molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been used for genetic analysis and the construction of genetic linkage maps (Gogorcena et al., 1993; Warburton and Bliss, 1996; Ortiz et al., 1997; Bartolozzi et al., 1998; De Vicente et al., 1998; Casas et al., 1999). On the downside, these DNA markers either present low reproducibility (RAPDs), or are time consuming and expensive (RFLPs, AFLPs). In the last years, microsatellite markers (SSR) are becoming the appropriate marker for molecular characterization and genetic diversity studies (Fang and Roose, 1997; Alvarez et al., 2001; Huang et al., 2002; Rallo et al., 2003; Moussaoui, 2005). Consisting of tandem repeats of mono-, bi-, tri- or tetra-nucleotides in the eukaryotic genome, these molecular markers present a very high polymorphism based on the number of the nucleotide motif repeats (Morgante and Olivieri, 1993). Besides polymorphism, they have a codominant

inheritance, they are reproducible and easily detected by PCR. Microsatellite marker primer pairs have been developed in peach (Sosinski et al., 2000; Testolin et al., 2000; Aranzana et al., 2002; Dirlewanger et al., 2002), and then used in genetic diversity analyses for sweet cherry (Dirlewanger et al., 2002), peach (Aranzana et al., 2003), and apricot (Romero et al., 2003; Zhebentyayeva et al., 2003).

'Miraflores' is a Spanish native peach variety, with attractive characteristics, especially high yields and good quality of fruits (Moreno, 2005). This nonmelting and clingstone variety is widely used for the fresh market as well as for processing. This variety appeared in 1970's at the Jalón valley (Aragón), one of the most important deciduous fruit tree growing areas in Spain. 'Miraflores' has unknown parents, although it is believed that it could originate from 'Campiel' seedlings, a traditional variety locally cultivated in this area (De Asso, 1798). The correct molecular characterization of this variety should prevent all confusion with other varieties with similar morphological characteristics, and enhance its use in peach breeding programs. On the other hand, this will be useful to warrant this variety as a specific typical product with a designation of origin.

Therefore, the aim of this study was to characterize nineteen clones of 'Miraflores' peach variety with unknown pedigree, using 20 SSR primer pairs. Ten old Spanish peach varieties originated from the same region were also included in this investigation, to check genetic similarities and possible genetic relationships with 'Miraflores'.

3-2. MATERIALS AND METHODS

3-2-1. Plant material

Thirty peach accessions, being either clones of 'Miraflores' or closely related varieties with other traditional peach varieties were collected throughout Aragón, and were analysed in this study. Nineteen accessions (clones and/or closely related varieties) were provided from a recent survey of 'Miraflores' variety ('Miraflores' 1 to 19). The other eleven accessions are old Spanish peach varieties originated from the same region of Aragón (Spain); two clones of 'Tipo Campiel' variety ('Tipo Campiel 1' and 'Tipo Campiel 2') and nine traditional cultivars ('Amarillo de Calanda', 'Zaragozano Amarillo', 'Amarillo de Gallur', 'Tardío del Pilar', 'Oropel', 'Zaragozano Rojo',

'Maluenda', 'Bonet IV', 'Amarillo de Septiembre'). From all accessions, young leaves were collected for DNA extraction.

3-2-2. Genomic DNA extraction

Fresh young leaves were ground to powder with liquid N_2 using a mortar and pestle. Genomic DNA was isolated from leaf samples using a CTAB (cethyltrimethyl ammonium bromide) extraction method (Cheng et al., 1997). The extraction buffer contained 2% CTAB, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris pH 8.0, and 0.4% 2-mercaptoethanol. Samples were incubated at 65ºC for approximately 30-60 min, mixed with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was transferred to a clean microcentrifuge tube and treated with RNAse A (10 mg/ml, 60 min, 37ºC), then mixed with an equal volume of cold isopropanol. The DNA was pelleted by centrifugation at 13,000 rpm for 3-5 min. The supernatant was removed and the DNA pellet washed with 500 µl of 70 % ethanol and 50 µl of wash buffer (3 M sodium acetate, 0.1 M magnesium acetate, pH 8.0), to remove residual CTAB, salt, and other contaminants. The pellet was dried for 1 h and then dissolved in 100 µl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). DNA quality was examined by electrophoresis in 0.8% agarose and DNA concentration was quantified spectrophotometrically (Gene Quant, Amersham Pharmacia Biotech). The extracted DNA was diluted to 5 ng /µl with TE buffer and stored at -20ºC for PCR amplifications.

3-2-3. DNA amplification

Twenty SSR markers were studied (Table 3-1) using primer pairs previously developed for peach. Amplification reactions were carried out in a final volume of 15 µl containing 10 ng of template DNA, 1X reaction buffer (20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 8.8), 2 mM $MgCl_2$, 0.2 mM of dNTPs (50 µM of each) (Amersham Pharmacia Biotech), 0.15 µM of forward and reverse primers each, and 0.5 U of Tth DNA Polymerase (Biotools B and M Labs, S.A., Spain), overlaid with a drop of mineral oil (Sigma, St Louis, MO). The PCR amplifications were carried out on a Gene Amp 2700 thermocycler (Applied Biosystems) using the following temperature cycles: 1 cycle of 3 min at 95ºC, 35 cycles of 1 min at 94ºC, 45 s at the corresponding annealing

temperature (Table 3-1) and 1 min at 72ºC. The last cycle was followed by a final incubation for 7 min at 72ºC and the PCR products were stored at 4ºC before analysis. Two independent SSR reactions were performed for each DNA sample. The DNA amplification products were loaded on 5% polyacrilamide sequencing gels. Gels were run for 2h at 65 W. The gels were silver-stained according to the protocol described by Bassam et al. (1983). Fragment sizes were estimated with the 30-330 bp AFLP ladder (Invitrogen, Carlsbad, Calif.) DNA sizing markers, and analysed by the Quantity One program (Bio Rad, Hercules, CA).

3-2-4. Data analysis

The number of alleles per locus was counted from the gel profile analysis. The observed heterozygosity (Ho) was calculated for each locus as the number of heterozygous individuals over the total number of individuals analysed. The expected heterozygosity was calculated as $He = 1 - \Sigma p_i^2$, where p_i is the frequency of i^{th} allele (Nei, 1973). The power of discrimination was calculated as $PD = 1- \Sigma g_i^2$, where g_i is the frequency of i^{th} genotype (Kloosterman et al., 1993). Those parameters served to evaluate the information given by the microsatellite markers (Table 3-1).

Data were analyzed using the NTSYS-pc, version 2.1 program (Rohlf, 2000). A 0/0.5/1 (absence/allele in heterozygosity/allele in homozygosity) matrix was constructed. The genetic distances between pairs of varieties were estimated from the matrix with the SIMGEND module using the Nei coefficient (Nei, 1972). Cluster analyses were carried out using Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). The resulting cluster was represented as a dendrogram. A principal coordinate analysis based on the similarity matrix was also performed.

3-3. RESULTS

3-3-1. Microsatellite diversity

Spanish peach varieties were analysed with 20 SSRs. All the SSRs studied were single-locus and produced alleles that could be scored, with a total of 46 ranging from one to six per locus, with a mean value of 2.3 alleles per locus. Fourteen out of the 20

SSRs employed, were polymorphic and it was possible to distinguish unambiguously 16 peach genotypes from the 30 accessions studied. However, six SSR loci (CPPCT002, CPPCT005, CPPCT017, CPPCT030, UDP98-408 and BPPCT007) were monomorphic for the peach plant material evaluated (Table 3-1). Observed heterozygosity (Ho) ranged from 0 to 0.70 in CPPCT006, with a mean value of 0.18. The expected heterozygosity (He) ranged from 0, for the monomorphic SSRs, to 0.67 in BPPCT008, with a mean value of 0.29. The power of discrimination (PD) varied from 0, for the monomorphic loci, to 0.67 in BPPCT008, with an average value of 0.31. Differences were found in the number of genotypes identified per locus. This number varied between one unique genotype in the monomorphic loci, to 8 genotypes using BPPCT008 with a mean value of 2.75 (Table 3-1).

The five most polymorphic microsatellites were CPPCT028, UDP98-022, BPPCT001, BPPCT008, and BPPCT015 (Table 3-1), most of them showed the highest He and PD. These SSRs allowed the unambiguous discrimination of the same 16 accessions separated using all the SSRs studied. These five SSR primer pairs detected a total of 21 alleles, ranging from 3 to 6 alleles per locus and with a mean value of 4.2 alleles per locus. The expected heterozygosity mean value for these microsatellites was He = 0.53, and the power of discrimination mean value was PD = 0.54.

***Table 3-1**. List of the 20 SSR primers used in this study, size range, annealing temperature, number of alleles and variability parameters.*

Locus code	References	Size range (bp)	Ta (ºC)	N	Ho	He	#Genotypes	PD
CPPCT002	Aranzana et al. 2002	102	58ºC	1	0.00	0.00	1	0.00
CPPCT004	Aranzana et al. 2002	262-277	56ºC	2	0.03	0.10	2	0.13
CPPCT005	Aranzana et al. 2002	154	58ºC	1	0.00	0.00	1	0.00
CPPCT006	Aranzana et al. 2002	182-192	60ºC	2	0.70	0.52	3	0.46
CPPCT017	Aranzana et al. 2002	183	60ºC	1	0.00	0.00	1	0.00
CPPCT022	Aranzana et al. 2002	248-292	58ºC	3	0.00	0.13	3	0.13
CPPCT028	Aranzana et al. 2002	134-138	58ºC	**3**	0.00	**0.35**	3	**0.35**
CPPCT029	Aranzana et al. 2002	192-196	58ºC	2	0.00	0.43	2	0.43
CPPCT030	Aranzana et al. 2002	186	56ºC	1	0.00	0.00	1	0.00
CPPCT033	Aranzana et al. 2002	151-153	58ºC	2	0.10	0.09	2	0.18
BPPCT001	Dirlewanger et al. 2002	152-166	60ºC	**6**	0.03	**0.62**	7	**0.63**
BPPCT007	Dirlewanger et al. 2002	146	58ºC	1	0.00	0.00	1	0.00
BPPCT008	Dirlewanger et al. 2002	100-156	59ºC	**4**	0.67	**0.67**	8	**0.67**
BPPCT015	Dirlewanger et al. 2002	168-222	62ºC	**5**	0.03	**0.50**	6	**0.52**
BPPCT017	Dirlewanger et al. 2002	162-174	60ºC	2	0.57	0.54	2	0.57
BPPCT038	Dirlewanger et al. 2002	127-129	62ºC	2	0.67	0.45	2	0.45
UDP98-022	Testolin et al. 2000	124-138	64ºC	**3**	0.00	**0.52**	3	**0.52**
UDP98-025	Testolin et al. 2000	134-142	65ºC	2	0.13	0.44	3	0.54
UDP98-407	Testolin et al. 2000	174-198	60ºC	2	0.57	0.40	3	0.59
UDP98-408	Testolin et al. 2000	102	56ºC	1	0.00	0.00	1	0.00
Mean				**2.3**	**0,18**	**0.29**	**2.75**	**0.31**

Ta: annealing temperature; N: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; # Genotypes: different genotypes per locus; and PD: power of discrimination.

3-3-2. Cluster and principal coordinate analysis

The genetic distance (D) among the different genotypes studied using the 20 SSRs was reproduced in the Neighbor Joining (NJ) dendrogram (Figure 3-1), according to the original data obtained in the similarity matrix, and based on the additive genetic distances among the genotypes (Saitou and Nei, 1987).

The dendrogram generated from the NJ cluster analysis showed two main groups. The first group was composed of the two 'Tipo Campiel' clones, 'Tardío del Pilar', 'Amarillo de Septiembre' and all the 'Miraflores' clones but 'Miraflores 3', which was clustered out of the two main groups. The second group included the rest of the studied varieties. In the first group, fourteen 'Miraflores' clones ('Miraflores' 5 to 11 and 'Miraflores' 13 to 19) were clustered together with a null genetic distance among them. The 'Miraflores 1' and 'Miraflores 2' were closely related to the fourteen

indistinguishable 'Miraflores' clones with a genetic distance D = 0.07 and D = 0.12, respectively. However, 'Miraflores 4' and 'Miraflores 12' were clustered further away, at a genetic distance of D = 0.26 and D = 0.21, respectively. The two 'Tipo Campiel' clones were genetically identical with the 20 SSRs, and 'Miraflores 4' was closely related to them with a small genetic distance of D = 0.04.

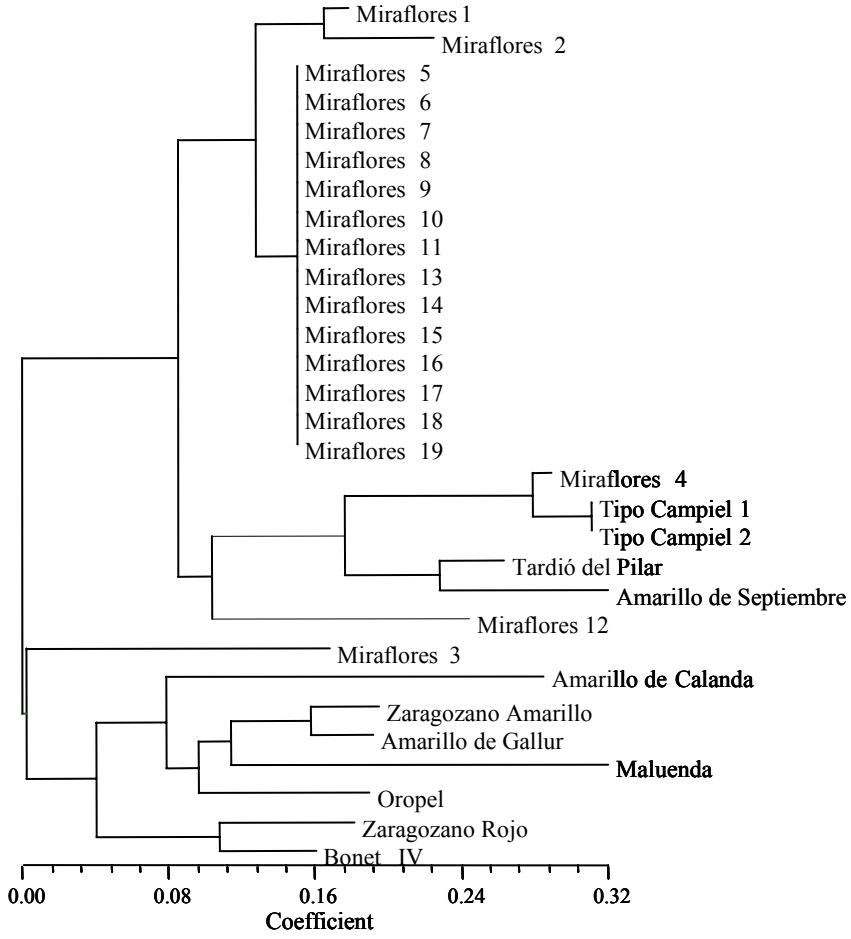

Figure 3-1. *Dendrogram of the 30 peach accessions obtained from the Neighbor Joining analysis (NJ) using Nei's genetic distance (1972) after amplification with 20 SSR primer pairs.*

The Principal Coordinate Analysis (PCA) generated two clearly significant components, which explained 44% and 32% of the total variance (Figure 3-2). This analysis showed well defined distribution patterns of the accessions, according to the genetic distances and the relationships among them. In the PCA, two main groups could

be clearly seen, the first one was composed by 'Amarillo de Septiembre', 'Tardío del Pilar' and all the 'Miraflores' clones but 'Miraflores 3' and 'Miraflores 4'. The second group included the varieties 'Oropel', 'Amarillo de Gallur', 'Zaragozano Amarillo' and 'Amarillo de Calanda'. Again, the PCA showed the small distance between the 'Miraflores 4' and the two 'Tipo Campiel' clones. The 'Miraflores 3' appeared further away from all the 'Miraflores' clones.

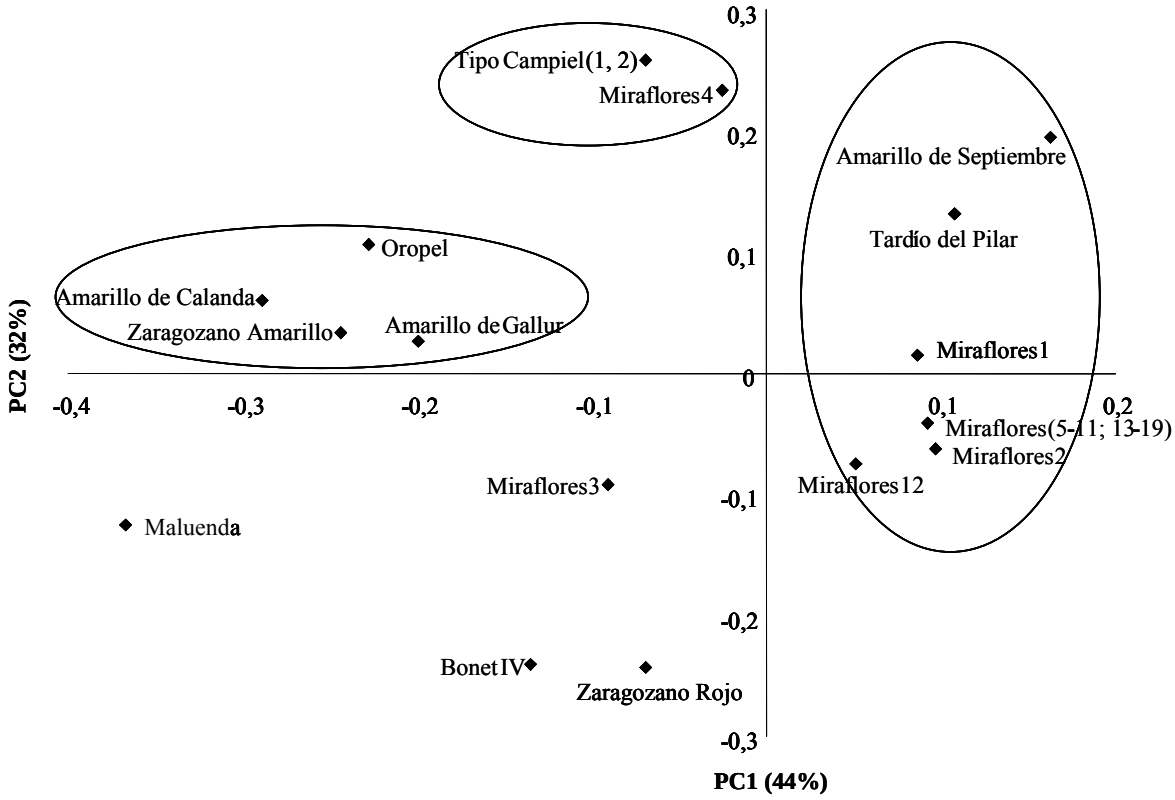

Figure 3-2. *Plot of the first two components (PC1 and PC2) of the principal coordinate analysis on the similarity matrix for 30 peach accessions (varieties and related clones) using 20 SSRs.*

3-4. DISCUSSION

3-4-1. Genetic diversity

The 20 SSR loci produced between 1 to 6 alleles with a mean value of 2.3 alleles per locus. This mean value calculated with 30 peach accessions, was lower than the mean values obtained by other authors in this species. Testolin et al. (2000) mentioned a value of 4.5 for a set of 50 varieties analysed with 26 microsatellites. Aranzana et al. (2002) cited 3.2 for a set of 25 varieties with 24 SSRs, and Dirlewanger et al. (2002) observed 4.2 for a set of 27 varieties with 41 SSR primer pairs. Mean values for the expected heterozygosity (He) and for the power of discrimination (PD) were also lower than the values recorded by these authors. In the present study, the He and PD were 0.29 and 0.31, respectively. Testolin et al. (2000) mentioned He = 0.47 and PD = 0.60; Aranzana et al. (2002) found value of 0.41 for He, and 0.60 for PD, and Dirlewanger et al. (2002) reported values of 0.41 for He, and 0.54 for PD. These results indicate a low variability in our plant material that can be explained by the close genetic relationship among the peach accessions studied and in particular, among the 'Miraflores' clones. However, using only the five most polymorphic primer pairs (BPPCT001, BPPCT008, BPPCT015, UDP98-022, and CPPCT028), the mean value of alleles per locus increased to 4.2. An increase in the mean values of the expected heterozygosity (He = 0.53) and the power of discrimination (PD = 0.54) has also been shown. These new values are closer to the ones mentioned by Testolin et al. (2000), Aranzana et al. (2002) and Dirlewanger et al. (2002). The high levels of polymorphism in the selected five SSRs, permitted to distinguish unambiguously 16 genotypes within the thirty studied accessions, the same genotypes that were discriminated using all the twenty SSR primers pairs. Thus, those five polymorphic SSRs are interesting markers for studies aimed at distinguishing highly related peach varieties. The fourteen clones of 'Miraflores' showed identical patterns for all the SSRs used in this study. These clones either belong to the same clone or represent different clones that differ by a single or few gene mutations, which could not be detected by SSR analysis (Testolin et al., 2000).

3-4-2. Cultivar relationships from cluster and principal coordinate analysis

The fourteen 'Miraflores' clones showed identical SSR profiles and were clustered together in the NJ tree. These clones have also similar pomological characteristics (M.A. Moreno, unpublished observation). The genetic similarity among some 'Miraflores' clones was also shown in a preliminary study reported by Moreno and Casas (2002) using 10 SSRs and 7 RAPDs. In terms of genetic distance, the 'Miraflores 1' and 'Miraflores 2' were closely related with the group of the fourteen identical 'Miraflores' clones with a genetic distance of 0.07 and 0.12, respectively. These two clones differed from the 'Miraflores' main group in only two SSR loci. The 'Miraflores 1' had a new allele with + 2 bp in the locus UDP98-022 (138 bp) and the lack of the allele 174 bp in the locus BPPCT017. The 'Miraflores 2' had a new allele with + 2 bp in the locus UDP98-022 (138 bp) and a new allele with + 2 bp in the locus CPPCT028 (136 bp). These two clones presented also similar *Rosaceae* flower shape, the same flowering and ripening date, and similar yield and fruit characteristics with the group of identical 'Miraflores' clones. The small molecular discrepancies of these two clones from the main group of 'Miraflores' may have been caused by SSR mutations as it was also suggested by Aranzana et al. (2003).

The 'Miraflores 12' is closer to the 'Miraflores' group ('Miraflores' 5-11 and 13-19) than to the old Spanish varieties at a genetic distance of D = 0.21. Moreover, this clone shared at least one allele in 17 SSR loci (34 alleles out of the 40 studied) with the group of identical 'Miraflores' clones. These results point out the existence of a close parental relationships between these clones and we can suggest that 'Miraflores 12' could be one of the 'Miraflores' progenitors. 'Miraflores 4' was clustered to the identical 'Miraflores' group at a genetic distance of D = 0.26. This clone is closely related to the two identical 'Tipo Campiel' clones at a genetic distance of D = 0.04. 'Miraflores 4' had at least one common allele in all SSR loci with the 'Tipo Campiel' clones and shared 37 alleles of the total alleles studied. According to the morphological data (data not shown), 'Miraflores 4' presents similar fruit characteristics to 'Tipo Campiel' cultivar (high percentage of redness in the skin), and similar flower morphology (bell flower shape). Moreover, the pulp close to the stone of the fruit is red as in the 'Tipo Campiel' fruits. Our findings confirm the hypothesis reported previously by Moreno and Casas (2002) that 'Miraflores 4' could be a seedling of 'Tipo Campiel' cultivar.

Finally, 'Miraflores 3' was clustered out of the two main groups shown in the NJ tree, at a genetic distance of D = 0.40. Furthermore, 'Miraflores 3' showed chemical and physical characteristics different from the rest of 'Miraflores' clones (fruit shape, yellow-red skin appearance, early ripening, sugar and acids content, etc.). This clone probably belongs to another variety which has morphological and phenological characteristics similar to those of 'Miraflores' variety. Previous work pointed out that this clone could be the 'Godina 58GC76' variety, based on similarities in fruit characteristics (fruit color, fruit shape, sugar and acid level etc.) (M.A. Moreno, unpublished observation).

The 'Miraflores' clones studied in this work have unknown parents and pedigrees. Old spanish varieties were included in the study to check an eventual relationship with 'Miraflores' clones. As it is shown in the dendrogram (Figure 3-1), and the PCA (Figure 3-2), the varieties 'Tardío del Pilar', 'Amarillo de Septiembre', 'Tipo Campiel' and all of the 'Miraflores' clones, but 'Miraflores 3', belong to the same group. The varieties 'Tardío del Pilar', 'Amarillo de Septiembre', and 'Tipo Campiel' were related to the 'Miraflores' clones at the genetic distances of 0.24, 0.30 and 0.31, respectively. In fact, these varieties, showed similar physical fruit characteristics (properties of the pulp and skin appearance) to those found in 'Miraflores' clones. Thus, we hypothesise that they could be close relatives of 'Miraflores' cultivar. However, the rest of the old Spanish varieties ('Amarillo de Calanda', 'Zaragozano Amarillo', 'Amarillo de Gallur', 'Oropel', 'Zaragozano Rojo', 'Maluenda' and 'Bonet IV'), were clustered in another main group far away from the 'Miraflores' clones.

In this work, the absence of SSR differences among fourteen clones of 'Miraflores' could be explained by the usual clonal propagation of 'Miraflores' carried out by nurseries and fruit growers. Another possibility could be that these clones may represent the original genotype of 'Miraflores' with few genetic variations such as sport mutations. However by using SSRs, it is highly improbable to distinguish mutants that differ from the original genotype in one or few genes (Botta et al., 1995; Moreno et al., 1998; Aranzana et al., 2003).

In conclusion, we regard the clones (1, 2, 5-11 and 13-19) of 'Miraflores' as true clones of the 'Miraflores' variety, and they will be included in future programs of selection. While 'Miraflores 12' was considered as one of the 'Miraflores' progenitors and 'Miraflores 4' could be considered as a new cultivar, closely related to 'Tipo Campiel'. 'Miraflores 3' is considered unrelated to the main group of 'Miraflores'.

These studies make possible the adequate choice of clones and varieties in new crosses for selection purposes.

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CAPÍTULO 4

Genetic analysis of introduced and local Spanish peach cultivars determined by SSR markers

ABSTRACT

A set of 94 peach cultivars including Spanish native peach and foreign commercial cultivars were analysed using 15 SSR markers, selected for their high level of polymorphism. The number of alleles obtained varied from two in the loci Pchgms1, pchgms2 and PceGA34, to eleven in UDP98-412 with an average of 6.73. The observed genotypes were ranged from two in pchgms2 to 24 in PS9f8 with a total of 185 genotypes in all loci studied and an average of 12 genotypes. All the cultivars studied showed a unique genetic profile, each one using the genotypic combination of all loci. The BPPCT001 was the most informative locus and also showed the highest discrimination power. Only six loci from the 15 SSRs used allowed the unambiguous separation of all the Spanish native cultivars included in this study, and the genotypic combination of only eight loci permitted the total differentiation of the 94 peach cultivars analysed. The six selected loci (BPPCT001, BPPCT006, BPPCT008, PS9f8, UDP98-022, and UDP98-412) seem to be very useful for future Spanish peach identification works, and will help to establish a molecular data base for native peach cultivars.

UPGMA analysis was performed from the genetic distance matrix, and allowed the arrangement of all genotypes according to their genetic relationships. The genetic diversity among cultivars, observed in this work, led to their separation according to their regional origin, their morphological characteristics and especially according to their fruit traits. The group of non-melting fruit flesh cultivars appeared more genetically variable than the rest of the cultivars tested. SSR markers have proved to be an efficient tool for fingerprinting peach cultivars and conducting genetic-diversity studies in peach.

Key words: Spanish native peach, Genetic relationship, Genetic diversity, SSR markers

4-1. INTRODUCTION

Today, the plant diversity is under threat as never before. In agriculture, the widespread adoption of a few improved varieties has narrowed the genetic base of most important food crops and led to the disappearance of hundreds of landraces (Gepts, 1995). Peach is one of the most economically important cultivated species of the *Prunus* genus subjected to intense breeding activity. Almost 500 new cultivars were released around the world during 1990-1996 (Fideghelli et al., 1998). The diversity of peach germplasm around the world has been drastically reduced, by the wide adoption of improved varieties which reduces genetic resources by displacing more diverse types. In Spain, the genetic diversity within peaches is also decreasing very rapidly, because of replacement of traditional varieties by introduced ones, mostly from North America (Badenes et al., 1998). In fact, United States cultivars are planted widely in many foreign countries and are being used in their breeding programs. On the other hand, genetic diversity in the United States peach cultivars is considered to be especially limited, because improved cultivars continue to be released from parents belonging to the same gene pool (Scorza et al., 1985).

The replacement of traditional varieties by American new cultivars is being stimulated by the exigency of the European market for melting-flesh cultivars that exhibit similar characteristics throughout the marketing season. In Spain, the peach industry is based on non melting flesh peaches, primarily derived from native populations which are genetically diverse and are an excellent source of traits for breeding. Characteristics such as medium and high chilling requirements, late flowering, extended harvest season from July to November, and very high flesh quality can be found in these populations (Badenes et al., 1998).

Managing peach biodiversity on farms and conserving it in collections of genetic resources are complementary approaches. Together they address the need for the continued future availability and use of peach biodiversity to improve productivity, both by breeding improved varieties and by improving farm ecosystems (Gepts, 1995). A detailed characterization of peach germplasm from Spain is necessary to save these important genetic resources and to obtain efficient collection. Genetic diversity, in particular, provides species with the ability to adapt to changing stresses, such as pests and diseases or drought.

Genetic markers reveal patterns and levels of genetic diversity that reflect the evolutionary relationships of individual accessions and can thus assist in identifying groups of accessions that are related by common ancestry (Gepts, 1995). The number of loci assessed should be as high as possible and preferably should be distributed at random in the genome to ensure adequate genome coverage (Aranzana et al., 2003a; 2003b).

Molecular and biochemical markers such as isozymes, seed proteins, RFLPs, RAPDs, AFLPs, SSRs, etc. have been used to characterise genetic diversity in *Prunus* germplasm collections (Messeguer et al., 1987; Gašíc et al., 2000; Hurtado et al., 2001; Quarta et al., 2001; Decroocq et al., 2004). In turn, this information can help in the selection of a core collection that is more representative of the main collection. Microsatellite markers, or simple sequence repeats (SSRs), which are codominant, and highly polymorphic markers easily detected with PCR procedure, appear as the best available choice of markers for peach assessment (Testolin et al., 2000; Dirlewanger et al., 2002; Aranzana et al., 2003a).

The principal aims of this study are the conservation and molecular characterization of the Spanish peach germplasm. For this purpose, several local germplasm prospecting were carried out in all the Spanish territory to start up collections of peach varieties (Cambra, 1988). These peach germplasm were morphologically characterised and now they are under molecular characterisation, to be efficiently conserved and used as source of traits for breeding programs.

In this work, 15 SSRs were used from published sequences for the screening of 94 peach accessions composed of Spanish peach cultivars, and some commercial varieties included as references, in order to: (1) examine SSR polymorphism among cultivars; (2) estimate the genetic relationships among native cultivated peaches; and (3) examine the genetic diversity among native and new commercial varieties.

4-2. MATERIALS AND METHODS

4-2-1. Plant material

A set of 94 peach [*Prunus persica* (L.) Batsch] cultivars including 64 Spanish native cultivars and 30 commercial cultivars, 26 from USA (Table 4-1), were studied.

Table 4-1. *Cultivars studied and their collections, origin, and main fruit characteristics.*

Cultivar	Collection[a]	Origin[b]	Fruit type	Color flesh	Flesh type
Alcañiz 1	EEAD	Teruel, SP	Peach	Yellow	Non-melting
Alcañiz 2	EEAD	Teruel, SP	Peach	Yellow	Non-melting
Amarillo de Calanda*	EEAD	Huesca, SP	Peach	Yellow	Non-melting
Amarillo Tardío	EEAD	Zaragoza, SP	Peach	Yellow	Non-melting
Andora	EEAD	USA	Peach	Yellow	Non-melting
Andross	CITA	USA	Peach	Yellow	Non-melting
Armking	CITA	USA	Nectarine	White	Melting
Baby Gold 5	EEAD	USA	Peach	Yellow	Non-melting
Baby Gold 6	EEAD	USA	Peach	Yellow	Non-melting
Baby Gold 7	EEAD	USA	Peach	Yellow	Non-melting
Baby Gold 8	EEAD	USA	Peach	Yellow	Non-melting
Ballejo	CITA	Zaragoza, SP	Peach	White	Non-melting
Benasque	EEAD	Huesca, SP	Peach	Yellow	Non-melting
Binaced	CITA	Huesca, SP	Peach	White	Non-melting
Blanco Tardío	CITA	Zaragoza, SP	Peach	White	Melting
Bonet 2	EEAD	Lérida, SP	Peach	Yellow	Non-melting
Bonet 3	EEAD	Lérida, SP	Peach	Yellow	Non-melting
Bonet 4*	EEAD	Lérida, SP	Peach	Yellow	Non-melting
Bonet 5	EEAD	Lérida, SP	Peach	Yellow	Non-melting
Borracho de Jarque	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Brasileño	EEAD	Murcia, SP	Peach	Yellow	Non-melting
Calabacero Candelo	CITA	Murcia, SP	Peach	Yellow	Non-melting
Calabacero Deleite	CITA	Murcia, SP	Peach	Yellow	Non-melting
Calabacero Rancho	CITA	Murcia, SP	Peach	Yellow	Non-melting
Calabacero Rincón	CITA	Murcia, SP	Peach	Yellow	Non-melting
Calabacero Soto	CITA	Murcia, SP	Peach	Yellow	Non-melting
Calanda	CITA	Teruel, SP	Peach	Yellow	Non-melting
Campiel Montes de Cierzo	CITA	Navarra, SP	Peach	Yellow	Non-melting
Campiel Rojo	EEAD	Huesca, SP	Peach	Yellow	Non-melting
Carolyn	EEAD	USA	Peach	Yellow	Non-melting
Carson	EEAD	USA	Peach	Yellow	Non-melting
Corona	EEAD	USA	Peach	Yellow	Non-melting
Cristalino	CITA	ARG	Peach	White	Melting
Del Gorro	EEAD	Zaragoza, SP	Peach	Yellow	Non-melting
Duraznillo	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Escolapio	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Everts	EEAD	USA	Peach	Yellow	Non-melting
Fantasia	EEAD	USA	Nectarine	Yellow	Melting
Early Diamond	EEAD	USA	Nectarine	Yellow	Melting
Flamekist	EEAD	USA	Nectarine	Yellow	Melting
Flavortop	EEAD	USA	Nectarine	Yellow	Melting
Fraga	EEAD	Huesca, SP	Peach	White	Non-melting
Gallur	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Garau	CITA	USA	Peach	Yellow	Non-melting
Gaume	CITA	USA	Peach	Yellow	Non-melting
Goiri	EEAD	Bilbao, SP	Peach	Yellow	Non-melting
Golden Queen	EEAD	NZL	Peach	Yellow	Non-melting
Halford	EEAD	USA	Peach	Yellow	Non-melting

***Table 4-1** (continued)*

Cultivar	Collection[a]	Origin[b]	Fruit type	Color flesh	Flesh type
Infanta Isabel	EEAD	Valencia, SP	Peach	Yellow	Non-melting
Jerónimo	CITA	Murcia, SP	Peach	Yellow	Non-melting
Jerónimo de Alfaro	EEAD	Murcia, SP	Peach	Yellow	Non-melting
Jerónimo Ortiz	CITA	Murcia, SP	Peach	Yellow	Non-melting
Jerónimo Torres	CITA	Murcia, SP	Peach	Yellow	Non-melting
Jesca	CITA	Teruel, SP	Peach	Yellow	Non-melting
Jungerman	EEAD	USA	Peach	Yellow	Non-melting
Klamt	EEAD	USA	Peach	Yellow	Non-melting
Loadel	EEAD	USA	Peach	Yellow	Non-melting
Maluenda*	EEAD	Zaragoza, SP	Peach	Yellow	Non-melting
Manolito	CITA	Lérida, SP	Peach	Yellow	Non-melting
Maruja Perfección	CITA	Murcia, SP	Peach	Yellow	Non-melting
May Grand	CITA	USA	Nectarine	Yellow	Melting
Miraflores Serapio	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Montamar	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Nuevo 2803	EEAD	FRA	Peach	Yellow	Non-melting
Ortiz	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Paloro A	EEAD	Badajoz, SP	Peach	Yellow	Non-melting
Paloro B	EEAD	Badajoz, SP	Peach	Yellow	Non-melting
Paraguayo Amarillo	CITA	Zaragoza, SP	Flat	White	Melting
Paraguayo Caspe	CITA	Zaragoza, SP	Flat	White	Melting
Paraguayo San Mateo	CITA	Zaragoza, SP	Flat	White	Melting
Paraguayo Sweet cap	CITA	FRA	Flat	White	Melting
Paraguayo Villamayor	CITA	Zaragoza, SP	Flat	White	Melting
Pepita	CITA	Huesca, SP	Peach	Yellow	Non-melting
Pigat	CITA	Huesca, SP	Peach	Yellow	Non-melting
Pigat Susagna	CITA	Huesca, SP	Peach	White	Non-melting
Pomar 1	CITA	Lérida, SP	Peach	White	Non-melting
Redhaven	CITA	USA	Peach	Yellow	Melting
Rojo Amarillo de Septiembre	CITA	Zaragoza, SP	Peach	Yellow	Non-melting
Rojo del Rito	CITA	Lérida, SP	Peach	Yellow	Non-melting
Rojo del Rito 5233	CITA	Lérida, SP	Peach	Yellow	Non-melting
San Jaime	CITA	Lérida, SP	Peach	Yellow	Non-melting
San Lorenzo	CITA	Huesca, SP	Peach	Yellow	Non-melting
Selma	EEAD	USA	Peach	Yellow	Non-melting
September Red	CITA	USA	Nectarine	Yellow	Melting
Starn	EEAD	USA	Peach	Yellow	Non-melting
Sudanell 1	CITA	Lérida, SP	Peach	Yellow	Non-melting
Sudanell 2	CITA	Lérida, SP	Peach	Yellow	Non-melting
Sudanell 3	CITA	Lérida, SP	Peach	Yellow	Non-melting
Sudanell Blanco	EEAD	Zaragoza, SP	Peach	White	Non-melting
Tambarria	CITA	Navarra, SP	Peach	White	Non-melting
Tardío del Pilar*	EEAD	Teruel, SP	Peach	Yellow	Non-melting
Valentín	CITA	Navarra, SP	Peach	White	Non-melting
Zaragozano Amarillo*	EEAD	Zaragoza, SP	Peach	Yellow	Non-melting
Zaragozano Rojo*	EEAD	Zaragoza, SP	Peach	Yellow	Non-melting

[a] EEAD: Estación Experimental de Aula Dei (CSIC); CITA: Centro de Investigación y Tecnología Agroalimentaria (DGA).
[b] SP: Spain; USA: United States of America; FR: France; ARG: Argentine; NZL: New Zealand
* Cultivars studied in Bouhadida et al., 2007.

All cultivars are clingstone but one ('Flavortop') which is freestone. Cultivars were obtained from the peach germplasm collections of the "Centro de Investigación y Tecnología Agroalimentaria" (CITA) and the "Estación Experimental de Aula Dei" (CSIC) both of them located at Zaragoza (Spain).

4-2-2. DNA extraction and microsatellite amplification

Total genomic DNA was extracted using the modified CTAB procedure described by Cheng et al. (1997). The DNA was quantified using a spectrophotometer (Gene Quant, Amersham Pharmacia Biotech), and diluted to 5 ng/µl to carry out PCR amplification reactions.

The 94 peach genotypes were analysed using 15 SSRs primer pairs previously developed in peach by different research groups (Table 4-2). These 15 SSRs were selected according to their high polymorphism and their clear and repeatable amplification patterns. Amplification reactions were carried out according to the protocol cited by Bouhadida et al. (2007). The PCR amplifications were carried out on a Gene Amp 2700 thermocycler (Applied Biosystems) using the following temperature cycles: 1 cycle of 3 min at 95ºC, 35 cycles of 1 min at 94ºC, 45 s at the corresponding annealing temperature (Table 4-2) and 1 min at 72ºC. The last cycle was followed by a final incubation for 7 min at 72ºC and the PCR products were stored at 4ºC before electrophoresis analysis. At least two independent SSR reactions were performed for each DNA sample until two data points were available for each SSR per cultivar combination. The DNA amplification products were loaded on denaturing 5% polyacrilamide gels. The gels were silver-stained according to the protocol described by Bassam et al. (1983). Fragment sizes were estimated with the 30-330 bp AFLP ladder (Invitrogen, Carlsbad, Calif.) DNA sizing markers, and analysed by the Quantity One program (Bio Rad, Hercules, CA).

4-2-3. Data analysis

To evaluate the information obtained with the 15 SSRs studied, we calculated the next parameters: the number of alleles (N), the effective number of alleles (N_e) per locus ($N_e = 1/\Sigma p_i^2$, were p_i is the frequency of the i^{th} allele), the observed heterozygosity (H_o = number of heterozygous individuals/number of individuals scored), the expected

heterozygosity (H_e = 1- Σp_i^2), and the wright's fixation index (F= 1-H_o/H_e) (Wright, 1965). The polymorphism information content (PIC) for each marker was also determined, using the following equation:

$$PIC = 1-\sum_{i=1}^{n} Pi^2-2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} P_i^2\, P_j^2\right]$$

Where p_i is the frequency of the i^{th} allele, and n is the number of alleles (Botstein et al., 1980).

The ability of a marker to discriminate between two random cultivars was estimated for each locus with the 'power of discrimination' (PD = 1-1/Σg_i^2, where g_i is the frequency of the i^{th} genotype) (Kloosterman et al., 1993).

A dendrogram was constructed from a 0/0.5/1 (absence/allele in heterozygosity/allele in homozygosity) matrix using the unweighted pair group method average (UPGMA) clustering. The genetic distance between cultivars was calculated according to the coefficient of Rogers (1972), and analyzed with the SIMGEND procedure of NTSYSpc V.2.1 program (Rohlf, 2000).

Table 4-2. *Characteristics of the 15 SSR markers studied.*

Locus	Ta (ºC)	Origin	References
BPPCT001	60	*P. persica*	Dirlewanger et al. (2002)
BPPCT006	58	*P. persica*	Dirlewanger et al. (2002)
BPPCT008	59	*P. persica*	Dirlewanger et al. (2002)
CPPCT005	58	*P. persica*	Aranzana et al. (2002)
CPPCT006	60	*P. persica*	Aranzana et al. (2002)
CPPCT022	58	*P. persica*	Aranzana et al. (2002)
CPPCT029	58	*P. persica*	Aranzana et al. (2002)
PceGA34	58	*P. cerasus*	Downey and Iezzoni (2000)
pchgms1	57	*P. persica*	Sosinski et al. (2000)
pchgms2	58	*P. persica*	Sosinski et al. (2000)
pchgms3	58	*P. persica*	Sosinski et al. (2000)
PS9f8	50	*P. cerasus*	Joobeur et al. (2000)
UDP98-022	64	*P. persica*	Testolin et al. (2000)
UDP98-407	60	*P. persica*	Testolin et al. (2000)
UDP98-412	57	*P. persica*	Testolin et al. (2000)

Ta: annealing temperature used

4-3. RESULTS

4-3-1. Genetic diversity of SSRs markers and cultivars identification

Ninety four peach cultivars were analysed with 15 polymorphic SSRs. All loci analysed in this study were multiallelic. The number of alleles detected for each locus was ranged from 2 (PceGA34, pchgms1, pchgms 2) to 11 (UDP98-412), with a total of 101 alleles for all loci and an average of 6.73 alleles per locus (Table 4-3). Allele sizes differed by two or more nucleotides. Size differences detected between alleles at the same locus, ranged from 2 to 68 bp and differences between consecutives alleles ranged from 2 to 32 bp, being 2 bp in 47% of the cases. The alleles obtained in different loci and their respective frequencies are shown in the Table 4-3.

The most frequent alleles of this study were detected for the loci pchgms1 (194 bp) and pchgms2 (157 bp), which showed frequencies higher than 90%. Seven alleles (6.93%) showed frequencies between 0.40 and 0.90, and 16 alleles (15.84%) showed frequencies between 0.20 and 0.40. However, 45 alleles (44.5%) showed low frequencies (less than 5%) (Table 4-3).

Thirteen loci, from the 15 analysed, showed one to six rare alleles (frequency less than 5%) each one, with a total of 45 (Table 4-3). Thirty three from the 45 rare alleles were observed for the non-melting peach cultivars. Moreover, 14 from the 45 rare alleles were unique (unique allele, with frequency = 0.005) and most of them (10) were also detected in the non-melting peach group.

Among all the polymorphic alleles (101) observed in this study, 92 were found in the 79 non-melting peaches (forty one were specific for this group), 59 in the eight melting peaches and 44 alleles in the seven nectarines. The nectarine and the melting peach groups showed only one specific allele each one. On the other hand, 36 alleles were shared by the three groups, 15 were common to the non-melting and melting peach groups, and seven were found in both nectarine and melting peach groups.

Samples in which only a single allele per locus was detected, were considered homozygous genotypes instead of heterozygous with a null allele (that did not amplify) for the purpose of computing genetic diversity parameters. Assuming homozygosity, the result will be an overestimation of the allele frequency and an underestimation of heterozygosity values.

Table 4-3. *Allele size (AS) in base pairs and allele frequency (AF) observed for the 94 peach cultivars analysed with 15 SSR markers.*

	BPPCT001		BPPCT006		BPPCT008		CPPCT005		CPPCT006		CPPCT022		CPPCT029		PceGA34		pchgms1	
Letter code	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF
A	134	0.059	119	0.601	103	0.106	153	0.867	182	0.005	232	0.027	178	0.005	144	0.362	194	0.947
B	148	0.011	121	0.053	135	0.080	155	0.005	184	0.144	252	0.053	184	0.005	148	0.617	196	0.053
C	154	0.149	127	0.043	137	0.250	157	0.032	194	0.761	254	0.011	192	0.484				
D	156	0.117	129	0.027	139	0.074	161	0.005	196	0.011	282	0.043	194	0.154				
E	158	0.176	131	0.154	149	0.005	176	0.005	198	0.005	284	0.011	196	0.293				
F	160	0.122	133	0.005	155	0.053	178	0.064			288	0.138	198	0.021				
G	162	0.202	135	0.053	157	0.287	180	0.021			294	0.011						
H	164	0.011	137	0.043	159	0.096					296	0.394						
I	166	0.154	141	0.021	161	0.037					298	0.229						
J											300	0.064						
K																		

Table 4-3 *(continued)*

	pchgms2		pchgms3		PS9f8		UDP98-022		UDP98-407		UDP98-412	
Letter code	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF
A	157	0.984	173	0.005	152	0.011	127	0.346	184	0.261	102	0.005
B	165	0.005	175	0.043	154	0.021	133	0.011	186	0.011	116	0.005
C			181	0.633	156	0.181	135	0.096	206	0.059	118	0.016
D			204	0.069	160	0.048	137	0.223	208	0.410	124	0.016
E			206	0.229	162	0.032	139	0.150	210	0.213	126	0.117
F					164	0.059	141	0.138	218	0.021	128	0.059
G					166	0.261	143	0.011	236	0.016	130	0.293
H					168	0.271					132	0.388
I					170	0.027					134	0.069
J					172	0.005					136	0.011
K											138	0.011

The observed heterozygosity varied between 1% (pchgms2) and 43% (BPPCT006), with an average value of 23%. The expected heterozygosity ranged from 3% in pchgms2 to 85% in BPPCT001, with an average value of 57% (Table 4-4). The wright's fixation index (F) compares He with Ho, estimating the degree of allelic fixation, and ranged from 0.30 in BPPCT006 to 0.76 in CPPCT029, with an average value of 0.58.

The PIC values are equal or slightly lower than the expected heterozygosity, and are correlated with the corresponding Ne (effective number of alleles) values (Table 4-4). The most informative locus of this study was BPPCT001, with a PIC value of 0.84 and a Ne of 6.67, while the less informative one was pchgms2 with a PIC and Ne values of 0.03 and 1.03, respectively (Table 4-4).

Table 4-4. *Parameters of variability calculated for the 15 SSR markers in 94 peach cultivars.*

Locus	N	Ne	Ho	He	F	PIC	# Genotypes	PD
BPPCT001	9	6.67	0.41	0.85	0.51	0.84	22	0.93
BPPCT006	9	2.53	0.43	0.60	0.30	0.59	15	0.79
BPPCT008	9	5.51	0.27	0.82	0.68	0.81	21	0.87
CPPCT005	7	1.32	0.14	0.24	0.43	0.24	11	0.31
CPPCT006	5	1.67	0.15	0.40	0.63	0.40	5	0.50
CPPCT022	10	4.23	0.19	0.76	0.75	0.75	16	0.81
CPPCT029	6	2.91	0.16	0.66	0.76	0.63	9	0.74
PceGA34	2	1.95	0.19	0.49	0.61	0.44	3	0.62
pchgms1	2	1.11	0.06	0.10	0.37	0.10	3	0.16
pchgms2	2	1.03	0.01	0.03	0.66	0.03	2	0.04
pchgms3	5	2.18	0.27	0.54	0.51	0.51	8	0.70
PS9f8	10	5.49	0.31	0.82	0.62	0.80	24	0.89
UDP98-022	7	4.93	0.35	0.78	0.55	0.77	17	0.89
UDP98-407	7	3.51	0.30	0.71	0.58	0.69	11	0.81
UDP98-412	11	3.86	0.21	0.74	0.71	0.72	18	0.81
Mean	**6.73**	**3.24**	**0.23**	**0.57**	**0.58**	**0.55**	**12.33**	**0.66**
All loci	**101**	**48.89**					**185**	**1**

N: Number of alleles; Ne: Effective number of alleles; Ho: Observed heterozygosity; He: Expected heterozygosity; F: wright's fixation index; PIC: Polymorphism information content; PD: Discrimination power.

Table 4-4 also shows the discrimination power (PD) for each SSR locus. The highest discrimination power was observed in BPPCT001 (0.93), whereas pchgms2 showed the lowest one (0.04) with a mean value of 0.66. According to this last result, it was observed that all loci distinguished up 185 different genotypes. The selection of the five most polymorphic loci which revealed the highest number of different genotypes:

PS9f8 (24); BPPCT001 (22); BPPCT008 (21); UDP98-412 (18); and UDP98-022 (17), allowed us to distinguished unambiguously all the 64 Spanish native peach cultivars, with the exception of "Paraguayo Villamayor" and "Paraguayo San Mateo". To identify separately these two cultivars, an additional BPPCT006 marker was selected, which revealed distinct alleles between them.

An identification key was established to distinguish among the 64 Spanish native peach cultivars (Figure 4-1). The procedure consist at joining together the cultivars presenting the same genotype for one locus. Cultivars which showed different genotypes were separated. This process was repeated for the six selected loci until the separation of all the cultivars, as it is shown in Figure 4-1.

The six selected loci also allowed the identification of 92 genotypes from the 94 studied. Whereas, the cultivars "Halford" and "Gaume", as well as "Valentín" and "Garau", showed the same genetic profile each pair with the six SSRs. The loci CPPCT022 and CPPCT029 were also selected to separate between the first and the second pair of cultivars, respectively. Consequently, only eight from the 15 SSRs initially used, allowed the total identification of all (94) peach cultivars included in the study.

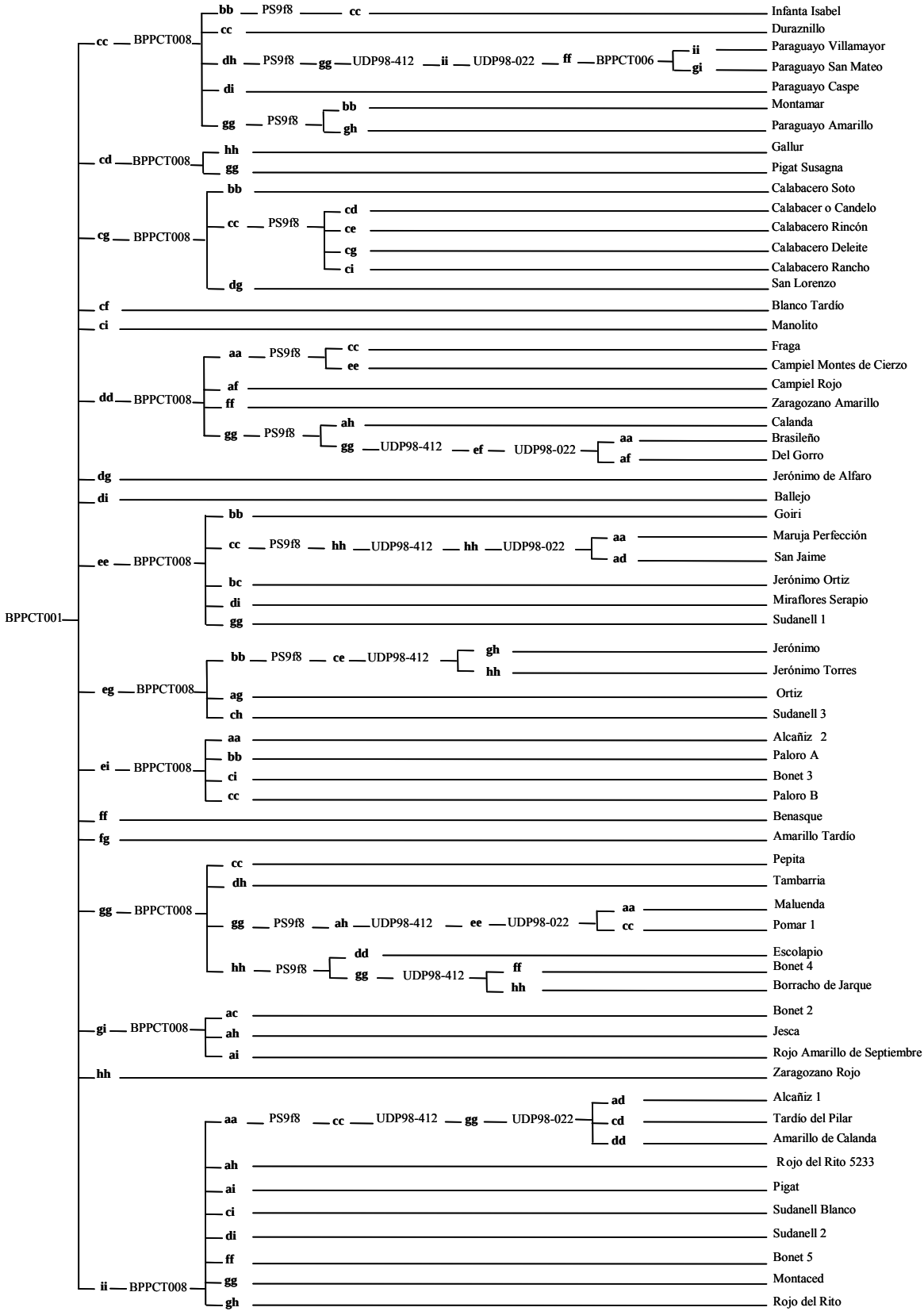

Figure 4-1. *Identification key for the 64 Spanish native peach cultivars based on six microsatellite markers: BPPCT001, BPPCT006, BPPCT008, PS9f8, UDP98-022 and UDP98-412.*

4-3-2. Genetic relationships among peach cultivars based on SSR variation

To elucidate genetic relationships among the peach cultivars studied, a dendrogram was produced using UPGMA cluster analysis and the Rogers distance over the 15 SSR loci (Figure 4-2).

The cultivars were clustered in two different groups. The first one constituted a main group which was subdivided into different subgroups. On the contrary, the second group of the dendrogram was composed of only five cultivars, representing four flat white melting peach and the white non-melting peach cultivar 'Binaced'.

At the similarity coefficient value of 0.38, the first main group, generated from the UPGMA cluster analysis, showed four subgroups. The first subgroup was composed of most clingstone non-melting peaches of this study, and this subgroup was also divided into different clusters. The second and third subgroups were composed of three clingstone non-melting peach cultivars each one, while all the nectarines and melting peaches (except Spanish flat melting peach cultivars) were clustered together in the fourth subgroup. This last one is divided into two clusters, the first one comprising all the nectarine cultivars, while the second one was composed of four melting peach cultivars and some non-melting peaches ('Ortiz', 'Sudanell 3', 'Montamar', 'Babygold 7', 'Babygold 6', and 'Babygold 8').

The first subgroup of the first main group appeared as the most diversified one, showing a large genetic variability among the peach cultivars. It is relevant to notice that inside of this subgroup, most of the peach cultivars are local Spanish one. We can also observe (Figure 4-2), that all peach cultivars from the Murcia region (South-West of Spain) were clustered together but one ('Brasileño'). Inside of this cluster, there are also peach cultivars from other regions of Spain (Valencia, Badajoz, Lérida, Bilbao) and some foreign ones. Inside of the first subgroup, it was also observed other clusters grouping Spanish native peach cultivars, mostly from the Ebro Valley area (Lérida, Zaragoza, Teruel, Huesca, Navarra) in the North-West of Spain.

Considering genetic distance values, non-melting peaches were the most diverse with an average distance between cultivars of 0.40, compared with 0.25 for nectarines and 0.32 for melting peaches. The non-melting peaches were also the most separated from the others, with average distances of 0.43 and 0.40 from the melting peaches and

nectarines, respectively. A lower average distance was detected between melting peaches and nectarines with a value of 0.35.

Some cultivars of this study were closely related, for example ‘San Jaime’ and ‘Maruja Perfección’; ‘Selma’ and ‘Calabacero Rancho’; ‘Amarillo de Calanda’ and ‘Tardío del Pilar’, with distance coefficient values of 0.020, 0.025, and 0.025, respectively. The highest distance between cultivars was found between ‘Paraguayo Villamayor’ and ‘Campiel Montes de Cierzo’ with a genetic distance of 0.58.

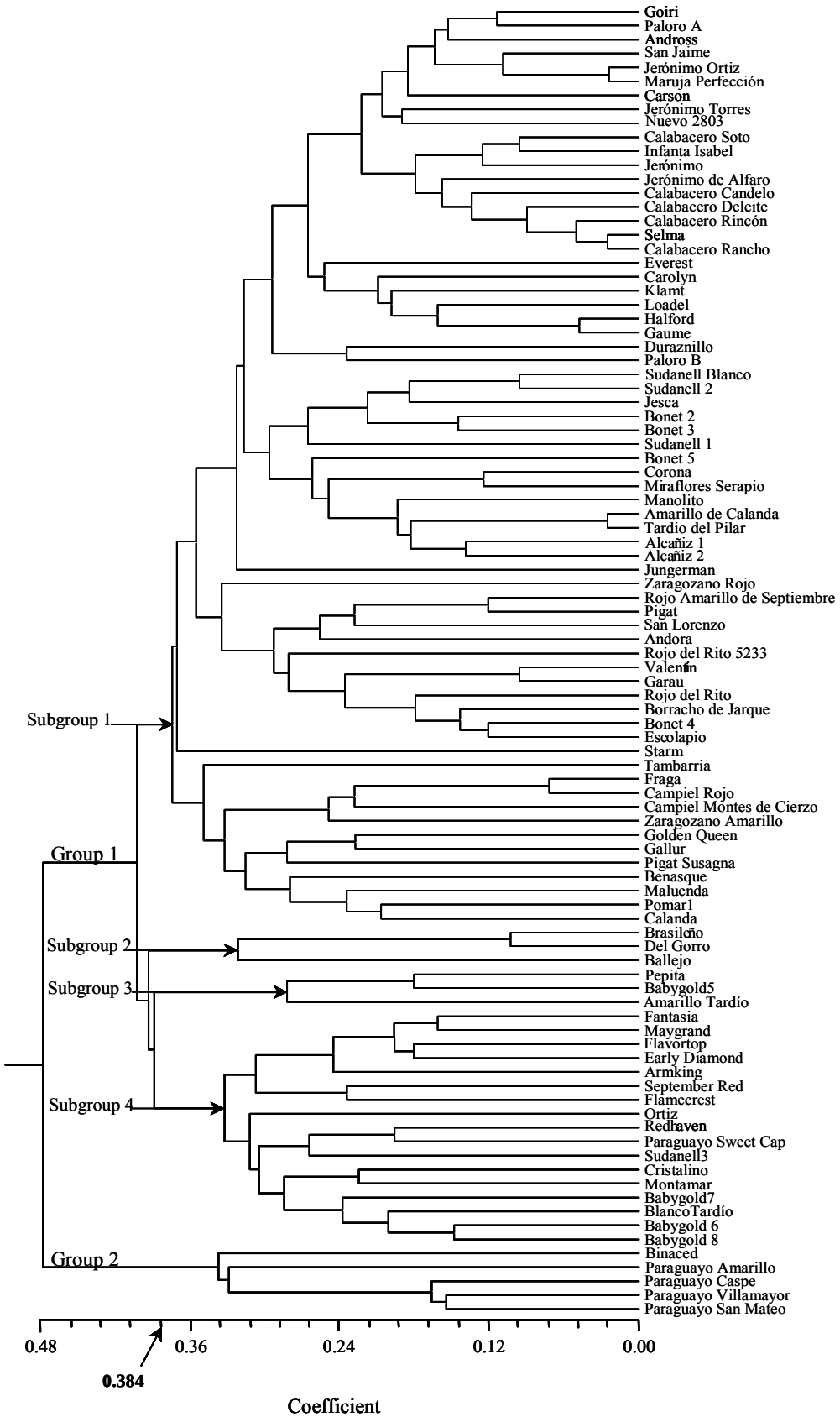

Figure 4-2. *UPGMA dendrogram of 94 peach cultivars based on their variation at 15 SSR loci.*

4-4. DISCUSSION

4-4-1. Microsatellite polymorphism

In this study, we obtained an average number of alleles per locus of 6.7. This average was higher than averages observed by others authors [(4.2) Dirlewanger et al., 2002; (4.5) Testolin et al., 2000; (2.3) Bouhadida et al., (2007)]. This can be explained by the use of a large number of cultivars and a set of high polymorphic microsatellites.

The level of polymorphism and the range of amplified band sizes (Tables 4-3 and 4-4) are similar to those reported by Aranzana et al. (2003a) for peach, using 11 common SSR markers to that used in this study. Results are also consistent with Testolin et al. (2000) using UDP markers in other peach cultivars.

Thirty three from the 45 rare alleles found in this study were observed for the non-melting peaches, 10 of them being unique alleles. This result can be explained by the high number of non-melting cultivars studied (79 out of 94), and can also give information about the genetic diversity inside of this peach group, especially inside of the Spanish non-melting peach group which represent 59 from the 79 non-melting peach cultivars of this study. Moreover, the non-melting peach cultivars presented 41 specific alleles out of the 92 detected for this group, which confirm its high variability with respect to nectarine (one specific allele) and melting peach (one specific allele) groups. Aranzana et al. (2003a) analysed peach cultivars, using 16 SSRs, and also observed that the non-melting peach group, was the most diverse one with 76 alleles, and showing the highest number (18) of specific alleles with respect to melting peach (8) and nectarine (16) groups. High average genetic distances detected first, within non-melting cultivars, and second, between non-melting and melting (0.43) as well as between non-melting and nectarine (0.40) groups, also give information about the high diversity of the non-melting peach cultivars of this study. These results well agree with results found by Aranzana et al. (2003a).

Values of the observed heterozygosity were lower than the corresponding expected heterozygosity for all loci. In the present study, observed heterozygosity averaged over the 15 SSR loci was 0.23, lower than the 0.35 mean value reported by Aranzana et al. (2003a) for a set of 212 peach cultivars analysed with 16 SSRs. This can be explained by the presence of null alleles that affected the heterozygosity level. The mean value of the expected heterozygosity was 0.57, slightly higher than the 0.50 obtained by

Aranzana et al. (2003a). The high allele number observed in this study (mean value=6.7) and the expected heterozygosity reflect the ability of SSR markers to provide unique molecular profiles for individual plant genotypes.

The highest PIC value and the highest Ne were observed for BPPCT001 (PIC=0.84 and Ne=6.67). Our results indicate that these two parameters, PIC and Ne, are very useful for the evaluation of adequate SSR markers to distinguish unambiguously related peach cultivars. In fact, it is important to select the most informative SSR loci to reduce the number of loci necessary to characterize a peach collection.

Moreover, the mean value of the discrimination power (PD) obtained in this study (0.66), agrees well with the mean value (0.64) obtained by Aranzana et al. (2003a), which also indicates that SSR markers can be used to identify a high level of variation in peach.

The fixation index average (F=0.58) indicated very great genetic differentiation (Hartl and Clark, 1997) among the peach accessions studied. At the same time, this average showed an excess of homozygotes (Murray, 1996). This may be due to two factors: First, 'null alleles' may be present which are leading to a false observation of excess homozygotes. Second, inbreeding may be common in the population.

Peach is, in fact, a self-fertile and naturally self-pollinates. It is considered tolerant of inbreeding, and open pollination usually results in less than 5% outcrossing (Fogle, 1977), which can explain this deviation from the Hardy-Weinberg equilibrium. On the other hand, the high level of genetic differentiation detected among the peach accessions could be explained by the peach mating system and by a low level of gene flow in this species. These findings could be attributing either to the different geographical region of accessions or to human impact. Maghuly et al. (2005) also reported similar observations for apricot.

4-4-2. Genetic relationships among cultivars

The dendrogram generated from the UPGMA cluster analysis (Figure 4-2) produced several significant groups, related to the morphological characteristics and the geographical origin of the genotypes. The dendrogram obtained shows two main groups, the first one constituted the most important group, which is divided in four subgroups subdivided in turn in clusters. The first subgroup is the largest one and was composed mostly of Spanish yellow and non-melting peach cultivars. Close

relationships among genotypes mostly coming from Murcia region (peaches of types 'Jerónimos', 'Maruja Perfección', and 'Calabaceros') were observed and were grouped under the same cluster, inside of the subgroup 1. The genetic relatedness among these cultivars can be explained by their same geographical origin. Inside of this cluster, there is also peach cultivars from different Spanish areas (Valencia, Badajoz, Lérida, Bilbao) and three foreign ones ('Andross', 'Carson', and 'Selma') coming from American breeding programs. The close relationships observed in this case can be explained by the similar morphological characteristics shared by these peach accessions and may be they come from the same genetic pool.

The subgroup 1 was composed of other clusters grouping Spanish native peach cultivars, mostly from the Ebro Valley (Lérida, Zaragoza, Teruel, Huesca, Navarra). The low genetic variability observed in each cluster of the subgroup 1 can be explained by the narrow genetic base explained by the common development of these varieties for the more narrowly defined environments of the Ebro Valley. Bouhadida et al. (2007) also mentioned a close relationship among some cultivars included in this study and originated from the Ebro valley area. Nevertheless, the genetic distances inter-clusters which grouped peach cultivars from narrow geographical origin (Ebro Valley) are relatively high, which explain a genetic diversity among clusters. This can be due to the mating system in peach which reduce the gene flow and consequently increase the genetic differentiation. The higher diversity observed in the group of non-melting peach cultivars, was also reported by Aranzana et al. (2003a).

All the nectarine and melting peach cultivars but four flat melting peach were clustered together in the fourth subgroup. Aranzana et al. (2003a) also observed a narrow genetic base among melting peach and nectarine with respect to non-melting peach cultivars. However, some non-melting peach cultivars ('Ortiz', 'Sudanell 3', 'Montamar', 'Babygold 7', 'Babygold 6', and 'Babygold 8') were also clustered in the fourth subgroup. This can be explained by the existence of a common genetic base within the cultivars of this subgroup. Aranzana et al. (2003a) also observed one non-melting peach cultivar ('Babygold 7') clustered with the melting peach group, which can agree with our findings.

The second main group of the dendrogram was composed of only five cultivars representing four flat melting peach and the white non-melting peach cultivar 'Binaced'. This group was clustered at a genetic distance of 0.48 from the first one. The relationship observed among 'Binaced' and Spanish flat peaches of the types

'Paraguayos' was also observed by Aranzana et al. (2003a) and Wünsch et al. (2005). These authors also showed the genetic separation of the Spanish flat peach group types 'Paraguayos' from the other peach cultivars.

4-4-3. Cultivar identification

Wünsch et al. (2005) examined 34 of the Spanish peach cultivars included in this study with 10 SSR markers. They observed various synonyms among them. In fact, they mentioned that the followed group of cultivars: 'Paraguayo Villamayor' and 'Paraguayo San Mateo'; 'Sudanell 2' and 'San Lorenzo'; 'Pomar 1' and 'Calanda'; 'Rojo del Rito (5233)', 'Escolapio' and 'Borracho de Jarque'; finally, 'San Jaime', 'Calabacero Candelo', 'Calabacero Rincón', 'Calabacero Soto', 'Calabacero Deleite', 'Calabacero Rancho' and 'Maruja Perfección' showed the same SSR profile patterns for each group. It is relevant to notice that only six SSRs of the present study permit to distinguish unambiguously among these accessions, and no synonyms were detected in this work. This can be explained by the high level of polymorphism of the SSRs selected and the use of polyacrilamide electrophoresis methods, which present a higher and better resolution and permit to detect more alleles present in the collection.

An identification key has been elaborated for the 64 Spanish native peach cultivars basing on the selected six SSRs (Figure 4-1). This method seems to be very useful for detection of homonyms and synonyms in the Spanish peach germplasm and aid to establish a molecular data base for Spanish native peach cultivars. Moreover, the most polymorphic microsatellites selected for the elaboration of this identification key can be used for future Spanish peach identification works.

Similar identification keys have been obtained for 49 date palm cultivars (*Phoenix dactylifera* L.) using three SSRs, which revealed 25 alleles and 57 genotypes (Zehdi et al., 2004), and for 54 Tunisian apricot cultivars based on five microsatellite primer pairs, revealing 28 alleles and 54 genotypes (Krichen et al., 2006).

4-5. CONCLUSION

Native non-melting peach population from Spain are genetically more diverse than North American melting peaches, which used a limited number of parents from the same gene pool inducing the drastically reduction of the genetic variability of the

commercial cultivars. Thus, Spanish non-melting peach cultivars can be an excellent source of traits for breeding.

To achieve certain breeding goals in peach, particularly for wider ecological adaptation, disease resistance and novel fruit quality traits, the use of germplasm from different groups and eco-geographical regions will be necessary.

The use of molecular markers appears very important for genotyping of the accessions, the classification and management of peach collections. Results from this study indicate that SSRs are excellent co-dominant markers for cultivars characterization in peach collections that can be used in plant breeding programmes.

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CAPÍTULO 5

Molecular characterization of *Prunus* rootstocks using microsatellite markers

ABSTRACT

Twenty microsatellite primer pairs, previously developed in peach, were used to explore genetic relationships among 44 clones, representing three groups of rootstocks which are defined as: (1) Peach-based rootstocks (*Prunus dulcis-persica, P. persica* x *P. davidiana*); (2) Myrobalan - Marianna plums (*P. cerasifera,* and interspecific hybrids having *P. cerasifera* as a parent); and (3) Slow growing plums (*P. insititia*, *P. domestica*, and *P. domestica* x *P. spinosa*). Eighteen SSR markers, from the 20 initially used, were able to amplify polymorphic products for the Peach-based rootstocks and 13 common markers gave also polymorphism for the Myrobalan-Marianna and Slow growing plums groups. The Dice coefficient of similarity was calculated between all pairs of accessions and their genetic similarity represented by an UPGMA dendrogram and a principal coordinate analysis. The genetic diversity detected among the 44 clones studied divided them in three groups, which are in agreement with their current taxonomic classification and their morphological characteristics. A more detailed analysis was carried out within the peach-based group using 18 SSRs. The results of this work suggest that SSR markers are transferable between species, and are valuable tools for identification of cultivars and analysis of diversity in *Prunus* species.

Key words: Genetic diversity, Molecular characterisation, *Prunus* rootstocks, SSR markers.

5-1. INTRODUCTION

Prunus is a large diverse genus of woody plants which belongs to the subfamily *Prunoideae* of the *Rosaceae*, and it is composed of approximately 400 species of trees and shrubs (Rehder, 1940). *Prunus* is economically important because many species are sources of edible fruits (e.g., apricots, cherries, nuts (almonds), peaches, and plums), oil, timber and ornamentals (Lee and Wen, 2001). In addition, several species of *Prunus* (*P. dulcis* D.A. Webb; *P. persica* (L.) Batsch; *P. cerasifera* Ehrh.; *P. domestica* L.; *P. insititia* L.; etc.) are used as rootstocks.

There are many different types of rootstocks being used for *Prunus* on a worldwide basis (Rom, 1982; 1984). Each one has a particular set of advantages and limitations for the soils and climates where they are used. Some of them have wide areas of adaptation and are used in more than one country and geographic region, while others appear to be more narrowly adapted.

Rootstocks, the below-ground portions of fruit or ornamental trees, are critically important because they are responsible for water and nutrient uptake, resistance to soil-borne pathogens, tolerance to environmental stresses, etc. (Cummins and Aldwinckle, 1983; Layne, 1987). Many of the most important agricultural attributes of the trees as a biotic unit, such as vigour, blossom initiation, fruit set, fruit size, and fruit flavour, etc., may be substantially influenced by the rootstock (Dozier et al., 1984). Therefore, a good rootstock should be compatible with a broad range of scion cultivars, should be disease free, especially virus free, and adapted to a wide range of soil types, soil reaction, soil fertility, and soil moisture, etc. (Layne, 1987). It is unlikely that any single rootstock for *Prunus* will have all of these attributes. Nevertheless, it is highly desirable to incorporate as many of these traits as possible to increase usefulness and broaden areas of adaptation of the new *Prunus* rootstocks.

Studies to improve *Prunus* rootstocks are underway at Aula Dei Experimental Station for obtaining new stone fruit rootstocks, with specific adaptation to Mediterranean environments (Moreno, 2004). Effective control and utilisation of *Prunus* rootstocks in breeding programs, and *Prunus* management, depends upon accurate and unambiguous characterisation. Classical methods of identification and characterisation of cultivars in fruit trees were based on morphological, cytological or phytochemical traits, which presented some disadvantages like high susceptibility to environmental factors and low degree of polymorphism. Molecular markers based on

DNA are stable, detectable in all tissues and independent of environmental or seasonal conditions. Molecular analyses have been previously performed within genus *Prunus*, using isozyme (Mowrey and Werner, 1990), RFLP (Kaneko et al., 1986; Uematsu et al., 1991), RAPD (Gogorcena and Parfitt, 1994; Lu et al., 1996; Casas et al., 1999) and PCR-RFLP (Badenes and Parfitt, 1995), some aimed at the differentiation and characterization of commercial cultivars, and other at elucidating the relationship between taxa within the genus.

Microsatellite markers are becoming a useful tool for genotyping, germplasm characterization and fingerprinting, because of their high level of polymorphism, co-dominant inheritance, abundance in the genome and high reproducibility. Moreover, they are often transferable, i.e. microsatellite primers developed for one species can be used to detect polymorphism at homologous loci in others. In fact, SSR markers developed in peach were used to analyse the genetic diversity of apricot (*P. armeniaca* L.) (Romero et al., 2003), sour cherry (*P. cerasus* L.) (Sosinski et al., 2000), and sweet cherry (*P. avium* L.) (Dirlewanger et al., 2002). Likewise, SSR markers identified in sweet cherry, peach and sour cherry were used to study genetic diversity in black cherry (*P. serotina* Ehrh.) (Downey and Iezzoni, 2000).

The objectives of this work were (1) to characterize commercial and selected rootstocks from the breeding program at Aula Dei Experimental Station, and (2) to analyse the genetic diversity among the different interspecific hybrids and species of *Prunus* included in this work.

5-2. MATERIALS AND METHODS

5-2-1. Plant material

The forty-four genotypes used in this study were obtained from the stone fruit rootstock collection maintained at Aula Dei Experimental Station (Zaragoza, Spain). For practical purposes, the clones were divided into three groups, as shown in Table 5-1. This classification was based on previous knowledge of taxonomic and morphologic similarity among the clones. The groups were defined as: 1) Peach-based rootstocks, including twelve peach [*P. persica* (L.) Batsch.] x almonds [*P. dulcis* D.A. Webb], three *P. persica* (L.) Batsch. x *P. davidiana* (Carr.) Franch hybrids, and one *P. persica* (L.) Batsch. x *P. davidiana* (Carr.) Franch x *P. dulcis* D.A. Webb]; 2) Myrobalan-Marianna plums which included six *P. cerasifera* Ehrh. rootstocks, and five interspecific hybrids having *P. cerasifera* as a parent; and 3) Slow growing plums (after the denomination proposed by Bernhard and Renaud (1990), which included ten *P. insititia* L., five *P. domestica* L. rootstocks and one interspecific *P. domestica* L. x *P. spinosa* L. hybrid. In this group are included the "Pollizo" plums, apparently *P. insititia*, traditionally utilized in Murcia (Spain) as rootstock of peach, almond and apricot (Kester and Graselly, 1987). With the criteria stated above, it was not possible to assign 'Fereley-Jaspi' (*P. japonica* Thunb. x *P. spinosa* L.) to any of the mentioned groups. It was included in the second group since it shared some morphological characteristics similar with this group.

5-2-2. Genomic DNA extraction and amplification

Fresh young leaves were ground to powder with liquid N_2 using a mortar and pestle. Genomic DNA was extracted from leaf samples according to the protocol described in Casas et al. (1999).

Twenty SSR markers were studied, using primer pairs previously developed in peach (Table 5-2). Amplification reactions were carried out according to the protocol cited by Bouhadida et al. (2007). The PCR amplifications were carried out on a Gene Amp 2700 thermocycler (Applied Biosystems) using the following temperature cycles: 1 cycle of 3 min at 95ºC, 35 cycles of 1 min at 94ºC, 45 s at the corresponding annealing temperature (Table 5-2) and 1 min at 72ºC. The last cycle was followed by a final incubation for 7 min at 72ºC and the PCR products were stored at 4ºC before analysis.

Two independent SSR reactions were performed for each DNA sample. The DNA amplification products were loaded on denaturing 5% polyacrilamide gels. Gels were run for 2h at 65 W. The gels were silver-stained according to the protocol described by Bassam et al. (1983). Fragment sizes were estimated with the 30-330 bp AFLP ladder DNA sizing markers (Invitrogen, Carlsbad, Calif.), and analysed by the Quantity One software (Bio Rad, Hercules, CA).

5-2-3. Data analysis

The following parameters were calculated: number of alleles per locus, observed heterozygosity (H_o calculated as the number of heterozygous genotypes divided by the total number of genotypes) and expected heterozygosity ($H_e = 1 - \Sigma p_i^{2)}$, where p_i is the frequency of i^{th} allele (Nei, 1973). The power of discrimination was calculated as $PD = 1 - \Sigma g_i^2$, where g_i is the frequency of i^{th} genotype (Kloosterman et al., 1993). The H_o, H_e and the PD were calculated only for the Peach-based rootstocks group, which represented diploid genotypes. The ploidy level of the second group is diploid for all but the two Marianna clones which are triploids. The third group was composed by hexaploid *Prunus* species.

The presence (1) or absence (0) of amplified fragments was recorded for each cultivar. A similarity matrix was generated using the Dice coefficient (Nei and Li, 1979). Similarity data were processed through the unweighted pair group method (UPGMA) cluster analysis using the NTSYS program (Rohlf, 2000) and finally depicted in two dendrograms, one for all rootstocks studied and one for the Peach-based rootstocks group. The goodness of fit, measured by the cophenetic correlation of both cluster analyses, was estimated using Mantel test (Mantel, 1967). A principal coordinate analysis (Gower, 1966) on the similarity matrix was also performed.

Table 5-1. *Characteristics of rootstocks used in the study.*

No.	Rootstock	Species	Origin	References
Peach-based rootstocks				
1	Adafuel	*Prunus dulcis x P. persica*	Spain	Cambra (1990)
2	Adarcias	*P. dulcis x P. persica*	Spain	Moreno and Cambra (1994)
3	Albatarrech	*P. dulcis x P. persica*	Spain	EEAD*
4	Alcañiz	*P. dulcis x P. persica*	Spain	EEAD*
5	Calanda	*P. dulcis x P. persica*	Spain	EEAD*
6	Caspe	*P. dulcis x P. persica*	Spain	EEAD*
7	GF 557	*P. dulcis x P. persica*	France	Bernhard and Grasselly (1981)
8	GF 677	*P. dulcis x P. persica*	France	Bernhard and Grasselly (1981)
9	Herce 5	*P. dulcis x P. persica*	Spain	EEAD*
10	Logroño	*P. dulcis x P. persica*	Spain	EEAD*
11	Tamarite	*P. dulcis x P. persica*	Spain	EEAD*
12	Tauste	*P. dulcis x P. persica*	Spain	EEAD*
13	Barrier	*P. persica* x *P. davidiana*	Italy	De Salvador et al. (1991)
14	Cadaman	*P. persica* x *P. davidiana*	France-Hungary	Edin and Garcin (1994)
15	Nemaguard	*P. persica* x *P. davidiana*	U.S.A.	Layne (1987)
16	Nemared	*(P. persica* x *P. davidiana) x P. persica*	U.S.A.	Ramming and Tanner (1983)
Myrobalan-Marianna plums				
17	Adara	*P. cerasifera*	Spain	Moreno et al. (1995a)
18	Ademir	*P. cerasifera*	Spain	Moreno et al. (1995b)
19	Myrobalan 713 AD	*P. cerasifera*	Spain	EEAD*
20	Myrobalan B	*P. cerasifera*	U.K.	Okie (1987)
21	Myrobalan 29C	*P. cerasifera*	U.S.A.	Okie (1987)
22	Myrocal	*P. cerasifera*	France	Bernhard and Renaud (1990)
23	Myrobalan GF 3-1	*P. cerasifera* x *P. salicina*	France	Bernhard and Renaud (1990)
24	Marianna GF 8-1	*P. cerasifera* x *P. munsoniana*	France	Salesses (1977)
25	Marianna 2624	*P. cerasifera* x *P. munsoniana*	U.S.A.	Okie (1987)
26	Miral 3278 AD	*P. cerasifera* x *P. dulcis* ?	Spain	EEAD*
27	Ishtara	*(P. cerasifera* x *P. salicina) x (P. cerasifera x P. persica)*	France	Renaud et al. (1988)
28	Fereley-Jaspi	*P. japonica* x *P. spinosa*	France	Bernhard and Renaud (1990)

***Table 5-1** (continued)*

No.	Rootstock	Species	Origin	References
Slow growing plums				
29	Adesoto 101	*P. insititia*	Spain	Moreno et al. (1995c)
30	Alguazas	*P. insititia*	Spain	Cambra (1970)
31	GF 655/2	*P. insititia*	France	Bernhard and Grasselly (1981)
32	Montizo	*P. insititia*	Spain	Felipe (1989)
33	Monpol	*P. insititia*	Spain	Felipe (1989)
34	PM 105 AD	*P. insititia*	Spain	Moreno (1990)
35	PM 137 AD	*P. insititia*	Spain	Moreno (1990)
36	PM 150 AD	*P. insititia*	Spain	Moreno (1990)
37	Puebla de Soto 67	*P. insititia*	Spain	Cambra (1970)
38	St. Julien A	*P. insititia*	France	Okie (1987)
39	Brompton	*P. domestica*	U.K.	Okie (1987)
40	Constantí	*P. domestica*	Spain	Cambra et al. (1989)
41	Penta	*P. domestica*	Italy	Nicotra and Moser (1997)
42	Tetra	*P. domestica*	Italy	Nicotra and Moser (1997)
43	Torinel	*P. domestica*	France	Anonymous (1992)
44	Damas GF 1869	*P. domestica* x *P. spinosa*	France	Salesses et al. (1988)

* Non-released clones from the Aula Dei breeding program

***Table 5-2.** List of the 20 SSR primers used in this study, size range, annealing temperature, and level of amplification for all the groups studied.*

Locus code	References	Size range (bp)	Ta (ºC)	Peach-based rootstocks	Myrobalan-Marianna plums	Slow growing plums
CPPCT002*	Aranzana et al. 2002	92-108	58ºC	++	++	++
CPPCT004	Aranzana et al. 2002	254-266	56ºC	++	-	-
CPPCT005	Aranzana et al. 2002	122-158	58ºC	++	++	++
CPPCT006	Aranzana et al. 2002	166-220	60ºC	++	++	++
CPPCT017*	Aranzana et al. 2002	180-202	60ºC	++	++	++
CPPCT022*	Aranzana et al. 2002	214-306	58ºC	++	++	++
CPPCT028	Aranzana et al. 2002	120-148	58ºC	++	++	++
CPPCT029	Aranzana et al. 2002	-	58ºC	dr	-	-
CPPCT030	Aranzana et al. 2002	160-190	56ºC	++	++	++
CPPCT033*	Aranzana et al. 2002	135-167	58ºC	++	++	++
BPPCT001	Dirlewanger et al. 2002	124-195	60ºC	++	++	++
BPPCT007*	Dirlewanger et al. 2002	123-167	58ºC	++	++	++
BPPCT008*	Dirlewanger et al. 2002	-	59ºC	dr	-	-
BPPCT015*	Dirlewanger et al. 2002	164-258	62ºC	++	-	-
BPPCT017*	Dirlewanger et al. 2002	139-181	60ºC	++	-	-
BPPCT038	Dirlewanger et al. 2002	103-139	62ºC	++	-	-
UDP98-022	Testolin et al. 2000	113-139	64ºC	++	-	-
UDP98-025*	Testolin et al. 2000	101-159	65ºC	++	++	++
UDP98-407	Testolin et al. 2000	166-240	60ºC	++	++	++
UDP98-408	Testolin et al. 2000	100-106	56ºC	++	++	++

Ta: annealing temperature;

++ good amplification; - no amplification; dr: Bands difficult to resolve

* SSR markers from the 'genotyping set' proposed by Aranzana et al. (2003b) for *Prunus* genome

5-3. RESULTS AND DISCUSSION

Twenty SSR markers have been tested on 44 genotypes of *Prunus*, belonging to three taxonomic groups. The SSR primer pairs used were previously developed for peach by other authors (Table 5-2). Of the 20 primer pairs investigated, 18 generated good amplifications in the first group of rootstocks (Peach-based rootstocks), while 13 detected amplification products for the second and the third group (Table 5-2). The remaining primer pairs gave bands which are difficult to resolve, or did not give PCR products for the rootstocks studied in each group and were consequently not included in the analysis. According to our results, relatedness among *Prunus* species allowed the successful use of some SSR primers, developed previously in peach. Cross-species amplification of SSR markers among *Prunus* species was already pointed out by Dirlewanger et al. (2002). Zhebentyayeva et al. (2003) and Wünsch and Hormaza (2002) also used SSR primers developed for peach to evaluate genetic diversity in apricot and in sweet cherry, respectively. All these studies confirm the transportability of SSR markers across *Prunus* species though at different degree of amplification and level of polymorphism for each species. Nevertheless, Aranzana et al. (2003b) proposed a 24 SSR marker 'genotyping set' for *Prunus*. Nine of those markers were included in our study, but only six of them amplified the three sets of materials included (Table 5-2). Markers BPPCT015 and BPPCT017 amplified only the group of Peach-based rootstocks. The marker BPPCT008 gave very limited resolution and was excluded from the analysis, although this SSR had been reported by Dirlewanger et al. (2002) as a good marker to amplify different species of *Prunus* (almond, apricot, peach, European plum, Myrobalan plum, and sour cherry).

Two types of comparisons were carried out to assess the genetic diversity for the three groups of rootstocks studied: (1) comparison among groups, and (2) comparison within groups of rootstocks.

5-3-1. Genetic diversity among groups

Thirteen SSR primer pairs gave polymorphic bands for all the 44 clones studied, and were used for the analysis of diversity among rootstocks groups, while the remaining SSR primers were discarded.

The 13 selected primer pairs generated distinctive products in the range of 92-306 bp (Table 5-2). It is relevant to notice that three of the most informative markers across groups (BPPCT001, CPPCT022 and UDP98-407) (Table 5-3) allowed the unambiguous differentiation of all the clones studied. Previously, seven RAPD markers were selected by Casas et al. (1999) to separate among the same three rootstock groups, but they did not allow the separation between 'Alguazas' and 'Adesoto 101' clones. These results reveal the power of SSR markers with respect to RAPDs, and we can consequently propose the use of the three selected SSRs of this study in future programs of identification of *Prunus* rootstocks.

The similarity among the 44 genotypes included in this study is graphically represented by an UPGMA dendrogram (Figure 5-1). The dendrogram was used to estimate relationships among clones based on shared alleles across all rootstocks.

At the similarity coefficient of 0.16, two main groups were distinguished, the first one representing the Peach-based rootstocks and the second one composed of the Myrobalan-Marianna and the Slow growing plums. The distribution of the rootstocks in the dendrogram can be explained by the botanical classification of the genus *Prunus*. In fact, Peach-based rootstocks belong to the subgenus *Amygdalus*, while both the Myrobalan-Marianna and the Slow growing plum rootstocks belong to the subgenus *Prunophora.* Consequently, the molecular distribution and the botanical classification of the 44 rootstocks studied are in good agreement. At the similarity coefficient of 0.19, the group of *Prunophora* rootstocks was divided into two clear sub-groups, the Myrobalan-Marianna plums and the Slow growing plums. The molecular data agree in this case with the rootstock classification based on morphological characteristics.

According to the dendrogram (Figure 5-1), we are able to distinguish unambiguously all the 44 clones studied. Genetic similarities (GS) between pairs of rootstocks had an average of 0.35. The cophenetic correlation coefficient was 0.86 suggesting a good fit of the dendrogram with the similarity matrix.

The average genetic similarity within clusters was 0.41, while average values between clusters were smaller. The most distinct group was that of the Peach-based rootstocks, which presented average similarities of 0.16 and 0.15 with Myrobalan-Marianna plums, and Slow growing plums, respectively. The average similarity between the two *Prunophora* groups was also low (0.19) but slightly higher than the average similarities with the first group of Peach-based rootstocks. Casas et al. (1999) reported higher average similarities among the same group of rootstocks and especially

between Myrobalan-Marianna group and the Slow growing plums, using seven RAPD primers. This can be explained by the higher level of polymorphism of the SSR markers which allowed a more accurate genetic diversity estimate among rootstocks.

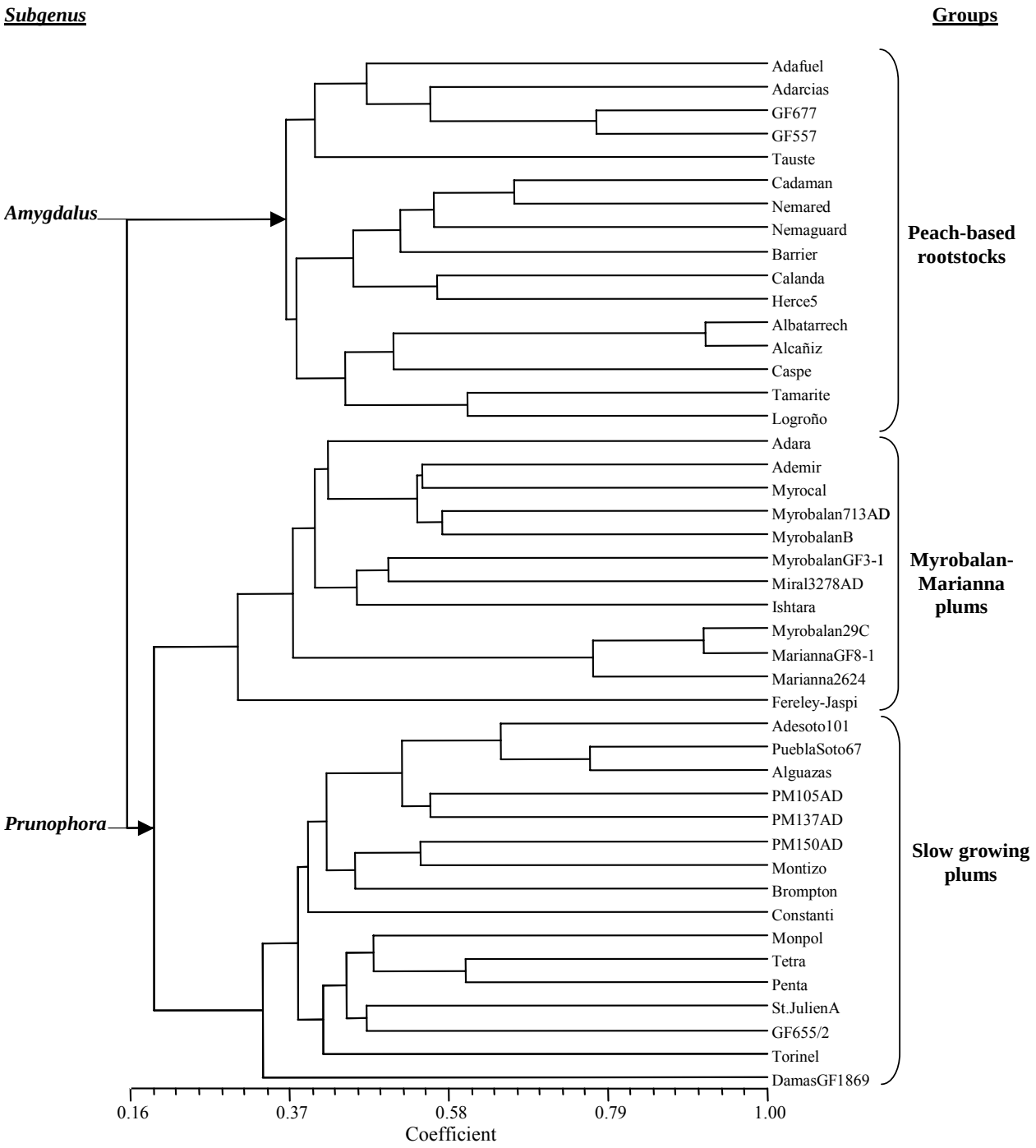

Figure 5-1. *Dendrogram of the 44 Prunus rootstocks obtained from the UPGMA cluster analysis using the Dice coefficient (Nei and Li, 1979), after amplification with 13 SSR primer pairs.*

The principal coordinate analysis (PCA) performed from the similarity matrix showed two significant axes, which explain 15% and 11% of the total variance respectively (Figure 5-2). The PCA agrees with cluster analysis and clearly establishes the distribution of rootstocks in three groups, which coincide with the Peach-based, Myrobalan-Marianna and Slow growing group rootstocks.

For the 44 rootstocks studied, it was possible to detect specific alleles for each group. In fact, 21 alleles distributed through 10 loci were specific to Peach-based rootstocks; 13 alleles, distributed through 6 loci, were private to Myrobalan-Marianna plums; and 26 alleles, distributed through 9 loci, were specific to Slow growing plums. These specific alleles can be used for future characterisation and identification of *Prunus* rootstocks or *Prunus* species. Myrobalan-Marianna and Slow growing plums shared 17 alleles distributed through eight loci. Eleven alleles distributed through six loci were common to Peach-based rootstocks and Slow growing plums. Finally, six alleles distributed through four loci were common to Peach-based rootstocks and Myrobalan-Marianna plums. The higher number of common alleles was observed between Myrobolan-Marianna and Slow growing plums, which can be explained by the close relationship among clones of these two groups. In fact, *P. domestica* and *P. insititia* (both hexaploids) were thought to be derived from a cross 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 and agrees with our findings.

The most distinct clones of this study were ‘Miral 3278 AD’, ‘Ishtara’, and ‘Fereley-Jaspi’. These clones are interspecific hybrids showing different agronomic characteristics and botanical descriptions with respect to the rest of clones (Table 5-1).

‘Miral 3278 AD’ is a natural hybrid of unknown parentage, whose morphology resembles Myrobalans, but it has some almond-like characteristics. Thus, a *P. cerasifera* x *P. dulcis* origin was postulated for this clone (Casas et al., 1999). In the present study, this clone showed common alleles with the Peach-based rootstocks that are also present in the Myrobalan-Marianna plums, but no specific alleles to Peach-based rootstocks were present in this clone. Our results obscure the hypothesis proposed above and support the hypothesis that the pedigree of ‘Miral 3278 AD’ includes other species besides *P. cerasifera*, but does not provide further clarification on their identity.

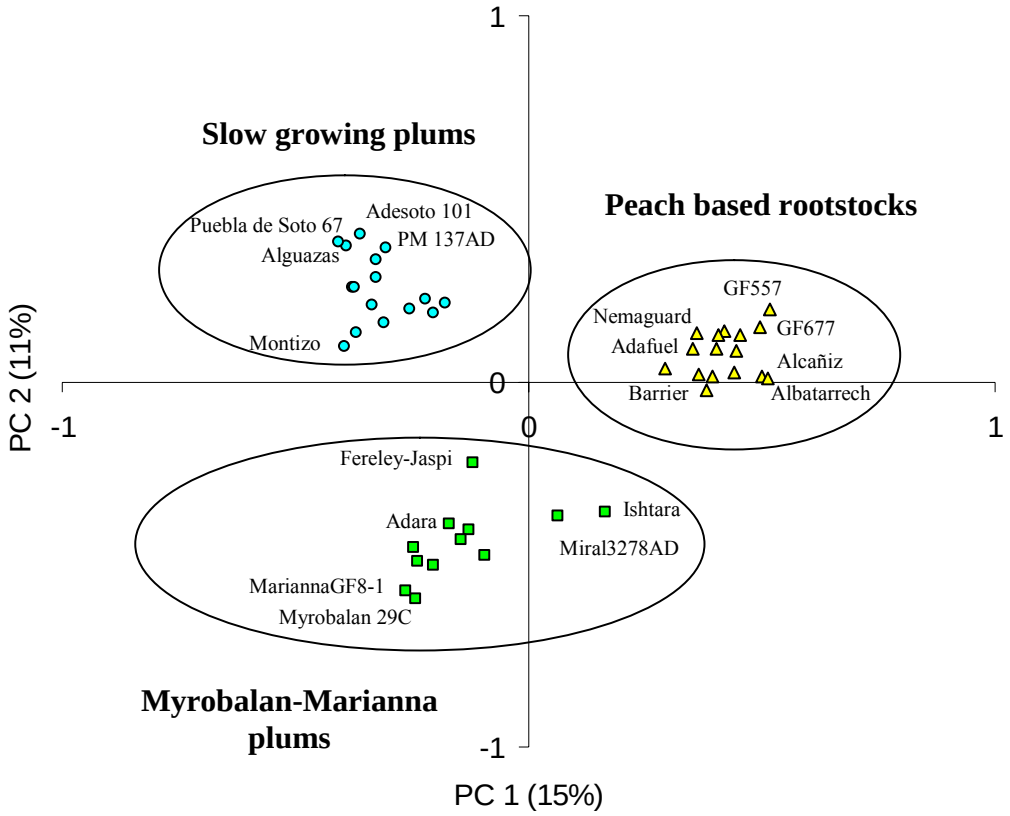

Figure 5-2. *Plot of the two first components (PC1 and PC2) of principal coordinate analysis on the similarity matrix for 44 Prunus rootstocks after amplification with 13 SSR primer pairs. Names of some relevant clones are shown in the figure.*

'Ishtara' is a complex interspecific hybrid, whose male parent was proposed to be a natural *P. cerasifera* x *P. persica* hybrid (Renaud et al., 1988). This rootstock shared two specific alleles (CPPCT033, 153 and UDP98-025, 143) with all hybrids of *P. persica* parentage, which were absent from all other plum rootstocks. Casas et al. (1999) also mentioned a band shared (AB9-18-1500) with all (but one) hybrids of *P. persica* parentage, which was absent from all other plum rootstocks using RAPD markers. These results confirm the possible presence of *P. persica* in the 'Ishtara' pedigree. Its position in the PCA (Figure 5-2), between the two groups of Peach-based rootstocks and Myrobalan-Marianna plums, but nearest to the second group, agrees with the double dose of *P. cerasifera* and the single dose of *P. persica* in the pedigree of 'Ishtara'.

'Fereley-Jaspi' (*P. japonica* x *P. spinosa*), was clustered with the Myrobalan-Marianna plums but at a lower level of aggregation (Figure 5-1). This clone presented an intermediate position between the two groups of plums with a genetic similarity of 0.35 and 0.21 with the Myrobalan-Marianna and Slow growing plums, respectively. The PCA (Figure 5-2) also showed the position of 'Fereley-Jaspi' between the two groups of plums. According to its pedigree, 'Fereley-Jaspi' is a hybrid between plum species belonging to the *Prunophora* subgenus, which could explain its relationship with other plum species of this study.

5-3-2. Genetic diversity within groups

Genetic diversity was detected among clones of each group of rootstocks. As mentioned before, 13 SSR markers gave polymorphic bands in all rootstocks studied. Moreover, other five SSRs were polymorphic for only the Peach-based rootstocks group.

We will describe first the diversity found within the two plum groups, amplified with 13 SSR markers, followed by the analysis of genetic diversity within Peach-based rootstocks, analysed with 18 SSRs.

5-3-2-1. Myrobalan-Marianna plum variation

The group of Myrobalan-Marianna plums included 12 clones of different species (Table 5-1). Thirteen SSR primers were used for the screening of this group and produced a total of 82 alleles, ranging from 2 (CPPCT017) to 10 (CPPCT005 and CPPCT006), with a mean value of 6.3 per locus (Table 5-3). The selection of the two most polymorphic loci which revealed the highest number of alleles for this group, CPPCT005 (10) and CPPCT006 (10), allowed us to distinguish unambiguously all the 12 clones of rootstocks group with the exception of 'Myrobalan 29C' and 'Marianna GF 8-1' which gave the same genetic profile. To identify these two cultivars, an additional CPPCT028 marker was selected which revealed distinct alleles between them.

In the dendrogram (Figure 5-1), it can be seen the clear separation of the two Marianna clones [*P. cerasifera* x *P. munsoniana* (Wight & Hedrick)] together with 'Myrobalan 29C' from the rest of the Myrobalan-Marianna clones. These three clones,

showed average genetic similarities of 0.82 among them. On the other hand, the rest of *P. cerasifera* clones were grouped together. The origin of Marianna rootstocks is not known. It has been postulated that they come from a natural hybrid between *P. cerasifera* and an American diploid species of *Prunus*, that is thought to be *P. munsoniana* (Crossa-Raynaud and Audergon, 1987). The position of 'Myrobalan 29C' in the dendrogram may seem as misclassification, according to its accepted denomination as Myrobalan (*P. cerasifera*). The morphology of 'Myrobalan 29C' presents similar characteristics than Marianna rootstocks. Grasselly (cited in Crossa-Raynaud and Audergon, 1987), also reported that 'Myrobalan 29C' was a Marianna seedling. Moreover, 'Myrobalan 29C', 'Marianna GF 8-1' and 'Marianna 2624' shared 4 alleles through 4 loci which were absent in the rest of the Myrobalan-Marianna plums studied, which support the high similarity found among these clones. Casas et al. (1999) also reported a very close relationship among 'Myrobalan 29C' and Marianna clones using RAPD primer pairs. This agrees with our findings and we can support the proposed hypothesis mentioning above that 'Myrobalan 29C' could be considered as a Marianna rootstock.

Table 5-3. *Number of loci and alleles observed for each rootstock group with the 13 SSR polymorphic among all the clones studied. These SSRs were used for the analysis of the diversity among groups.*

Primer code	**Peach-based rootstocks**		**Myrobalan-Marianna plums**		**Slow growing plums**	
	Loci number	**Alleles number**	**Loci number**	**Alleles number**	**Loci number**	**Alleles number**
CPPCT002	1	5	1	4	3	8
CPPCT005	1	2	1	10	2	16
CPPCT006	1	10	1	10	2	11
CPPCT017	1	5	1	2	1	1
CPPCT022	1	8	1	7	3	15
CPPCT028	1	3	1	7	2	10
CPPCT030	1	4	1	4	2	6
CPPCT033	1	9	1	8	2	8
BPPCT001	1	8	1	6	3	19
BPPCT007	1	8	1	7	3	12
UDP98-025	1	13	1	6	2	10
UDP98-407	1	7	1	9	2	15
UDP98-408	1	4	1	2	1	2
Total	**13**	**86**	**13**	**82**	**28**	**133**
Mean		**6.6**		**6.3**		**4.75**

5-3-2-2. Slow growing plums variation

This group included 16 hexaploid *Prunus* (*P. insititia*, *P. domestica*, and *P. domestica* x *P. spinosa*) clones (Table 5-1) and each primer used was able to detect from one to three loci (Table 5-3). Thirteen SSR primer pairs, from the 20 initially used, amplified a total of 133 polymorphic alleles through 28 loci detected in this group (Table 5-3) with a mean value of 4.75 alleles per locus. The primer BPPCT001 amplified a total of 19 alleles through the three loci detected in this case and allowed the unambiguous separation of all the 16 clones.

According to the dendrogram (Figure 5-1), *P. insititia* and *P. domestica* rootstocks did not form different clusters. This is not surprising, because both species have evolved in the same geographical area (Ramming and Cociu, 1990), and are inter-fertile (Crane and Lawrence, 1952). Natural hybridisation between *P. domestica* and *P. insititia* may have been favoured by the pollen self-incompatibility in both species (Bernhard et al., 1951).

'Adesoto 101', 'Puebla de Soto 67', and 'Alguazas' are very closely related, which is explained by the fact that they are 'Pollizo' collected in the same geographic area (South of Spain). On the other hand, the close genetic similarity (GS = 0.55) found between 'PM105AD' and 'PM137AD' can be explained by the fact that they are selected rootstocks coming from open pollinated pre-selected clones of 'Pollizo' and though they have been described as *P. insititia*, their male parent is not known. The clones 'PM 150 AD' and 'Montizo' also showed a close relationship (GS = 0.54) and both are selected rootstocks from open pollinated populations of 'Pollizo'. According to the Figure 5-1, we also observed a close relationships between two pairs of clones: 'Tetra' and 'Penta' (GS = 0.60), and 'St Julien A' and 'GF 655/2' (GS = 0.47). This result can be explained by the common geographic origin of each pair of clones (Table 5-1).

5-3-2-3. Peach-based rootstocks

Microsatellite diversity

In our set of Peach-based rootstocks, peach-derived SSR markers detected considerable polymorphism. Eighteen of the 20 SSR markers used in this study

produced a total of 124 polymorphic bands over the 16 screened clones of the Peach-based rootstocks group (Table 5-4).

Table 5-4. *Allele number and parameters of variability in the Peach-based rootstocks group with the 18 polymorphic SSRs.*

Locus code	Peach-based rootstocks alleles	Ho	He	#Genotypes	PD
CPPCT002	5	0.50	0.61	8	0.84
CPPCT004	5	0.00	0.73	4	0.73
CPPCT005	2	0.06	0.06	2	0.12
CPPCT006	10	0.88	0.76	7	0.88
CPPCT017	5	0.63	0.67	6	0.63
CPPCT022	7	0.69	0.76	9	0.87
CPPCT028	3	0.00	0.53	3	0.53
CPPCT030	4	0.38	0.32	4	0.55
CPPCT033	9	0.69	0.78	10	0.86
BPPCT001	8	0.50	0.55	8	0.71
BPPCT007	8	0.44	0.77	10	0.84
BPPCT015	11	0.00	0.87	10	0.87
BPPCT017	11	0.81	0.82	11	0.89
BPPCT038	8	0.69	0.64	9	0.84
UDP98-022	5	0.19	0.72	7	0.79
UDP98-025	12	0.62	0.79	12	0.92
UDP98-407	7	0.69	0.77	10	0.87
UDP98-408	4	0.50	0.67	6	0.82
Total	**124**			**136**	
Mean	**6.90**	**0.46**	**0.66**	**7.56**	**0.75**

Ho: observed heterozygosity; He: expected heterozygosity; # Genotypes: different genotypes per locus; and PD: power of discrimination.

The number of alleles observed at each locus ranged from 2 (CPPCT005) to 12 (UDP98-025), with an average of 6.9 (Table 5-4). This value obtained for only 16 clones is relatively high when compare it with values reported for other *Prunus* species considering the number of accessions studied. Aranzana et al. (2003a) observed a mean value of 7.3 for a set of 212 peach varieties analysed with 16 microsatellites. In our study, observed and expected heterozygosities averaged over the 18 SSR loci were 0.46 and 0.66, respectively (Table 5-4). These parameters are higher than the mean values reported for SSRs in peach (Aranzana et al., 2003a; Bouhadida et al., 2007). High allele number and high heterozygosity obtained in the present study reflect the ability of SSR markers to provide unique genetic profile for individual plant genotypes. This

discriminating ability is much greater with hybrid cultivars than with mutant or clonal cultivars, which cannot be distinguished unless mutations are located within SSR loci or flanking regions (Aranzana et al., 2003a). Observed heterozygousity was zero for three of the SSR (CPPCT004, CPPCT028 and BPPCT015). A simple explanation of these values is that the almond alleles didn't amplify due we are working with peach SSR. Similar cases were reported by Howad et al. (2005), where 13% of the primers used were monomorphic and dominant for the peach alleles. On the other hand, genetic diversity is higher in *Prunus* hybrids than in simple peach cultivars because the last ones are predominantly self-pollinating. Moreover, almonds (*P. dulcis*) is known to be self-incompatible and consequently more polymorphic than peach (Tamura et al., 2000, Xie et al. 2006). Higher values were reported for outcrossing species. Zhebentyayeva et al. (2003) and Maghuly et al. (2005) mentioned in apricot average values for H_o and H_e higher than in peach which well agree with our results. The highest number of different genotypes was registered for the locus UDP98-025 (12), and the lowest for CPPCT005 (2) (Table 5-4).

A total of 136 genotypes were observed for all loci, with an average of 7.5 genotypes per locus. The selection of the two most polymorphic loci, UDP98-025 and BPPCT017, allowed to distinguish unambiguously all the 16 clones of Peach-based rootstocks group, with the exception of 'Albatarrech' and 'Alcañiz' which gave the same genetic profile. To differentiate these two cultivars, an additional marker (BPPCT007) was selected, which revealed distinct alleles between them.

The average power of discrimination (PD=0.75) observed for Peach-based rootstocks is high and is comparable with the 0.64 mean value reported for peach by Aranzana et al. (2003a). Our findings indicate that peach SSR markers are very efficient to identify genetic variability among Peach based-rootstocks.

To elucidate genetic relationships among Peach-based rootstock clones, a dendrogram was produced using UPGMA cluster analysis and the Dice coefficient over 18 SSR loci (Figure 5-3). The dendrogram in Figure 5-3 showed a different distribution of clones with respect to the one shown in Figure 5-1. According to the new one, the genotypes studied can be divided into two main groups. The first group contained the four genotypes presenting the pedigree *P. persica* x *P. davidiana*, whereas the second group included the peach hybrids of the type *P. persica* x *P. dulcis*. All non-released rootstocks of EEAD collection (but 'Herce 5') were clustered together in a subgroup

(Figure 5-3). This separation of all the Peach-based rootstocks was in good agreement with their botanical classification. This suggests that a larger number of markers was necessary to lead to a more accurate genetic relationships among clones which respond truly to the morphological characteristics and the botanical descriptions. The cophenetic correlation coefficient was 0.8 suggesting a good fit of the dendrogram of the Figure 5-3 with the similarity matrix.

'Albatarrech' and 'Alcañíz' showed a very close relationship, as Casas et al. (1999) reported using RAPD markers. This is not surprising because these two clones come from the same region and present similar morphological characteristics. A high genetic similarity was also detected between 'Nemared' and 'Nemaguard' with a similarity value of 0.71. A close relationship was also mentioned by Lu et al. (1996) and Casas et al. (1999) between these two cultivars. This was expected, as 'Nemaguard' is one of the parents of 'Nemared' (Ramming and Tanner, 1983).

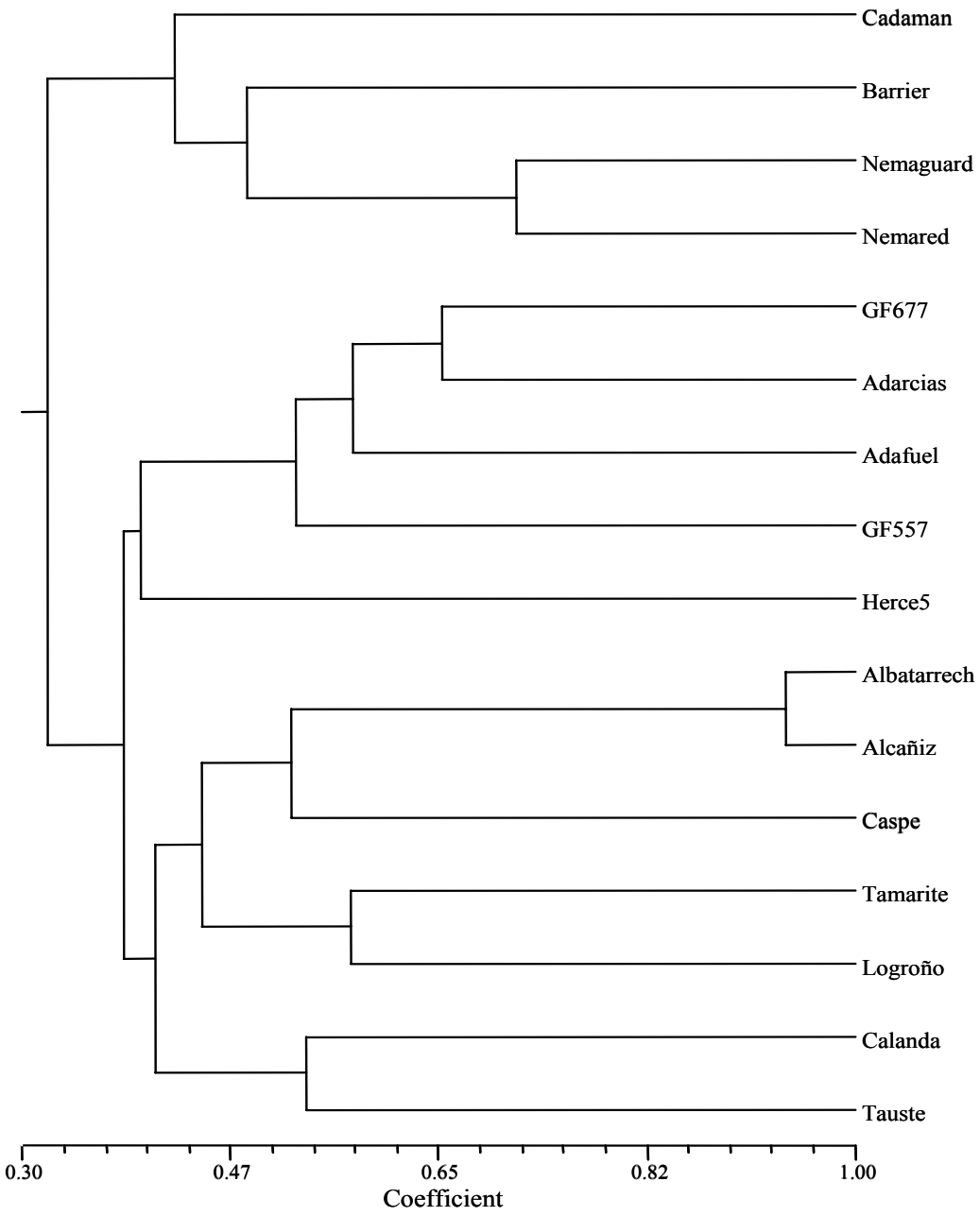

Figure 5-3. *Dendrogram of the 16 peach-based rootstocks obtained from the UPGMA cluster analysis, using the Dice coefficient (Nei and Li, 1979) after amplification with 18 SSR primer pairs.*

In summary, the analysis of genetic diversity among groups of *Prunus* rootstocks using peach-derived SSR markers, allowed us to cluster successfully the clones according to their morphological characteristics, and their botanical classification. In the present study, polymorphism observed among the rootstocks is large, since we have been able to distinguish them unambiguously using only three SSR markers from the 20 initially used. Moreover, transportability of microsatellite seems to be very efficient, this means that the microsatellite primers developed for one species (in this case for peach) can be used to detect polymorphism at homologous loci in other related species of the genus *Prunus*.

Molecular characterisation of *Prunus* rootstocks is of great interest for breeding programs. This will guaranty a correct identification of the rootstocks during the propagation process, without influence of environmental factors that may limit or influence phenotypic characterization. Moreover, the study of genetic diversity among different genotypes and populations is very useful to choose parental genotypes for crosses, to optimise germplasm management and to maximize diversity.

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CAPÍTULO 6

Chloroplast DNA diversity in *Prunus* and its implication on phylogenetic relationships

ABSTRACT

Chloroplast DNA (cpDNA) in 84 *Prunus* accessions (interspecific hybrids and *Prunus* species) were analysed to identify the maternal origin of the interspecific hybrids of *Prunus* and to establish phylogenetic relationships among *Prunus* species. PCR-RFLP (polymerase chain reaction-restriction fragment lengh polymorphism) method, which includes amplification of cpDNA regions with three universal primer pairs (K1K2, HK, DT) and subsequent digestion with three restriction enzymes (*Alu*I, *Hin*fI, *Taq*I), revealed 33 haplotypes for the 84 accessions studied. Fourteen from these cpDNA haplotypes were shared by two or more accessions, and 19 were private. The accessions sharing the same haplotype had maternal relationships among them, which allowed to confirm the identity of maternal progenitors of *Prunus* interspecific hybrids in these cases. UPGMA and maximum parsimony analyses were performed based on shared common fragments among the 33 haplotypes. The detected cpDNA polymorphisms enabled to reconstruct the phylogenetic relationships among the studied *Prunus* accessions. Most of the recovered relationships are in agreement with current taxonomic hypotheses and artificial crosses.

Key words: *Prunus*, PCR-RFLP, cpDNA haplotypes, phylogenetic analysis.

6-1. INTRODUCTION

Prunus (subfamily *Prunoideae* of the family *Rosaceae*) is a large and diverse genus comprised of approximately 400 species of trees and shrubs. This genus chiefly originated in the Northern hemisphere, 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 includes *P. spinosa* L. (2n=4x=32) and hexaploid species are represented by *P. domestica* and *P. insititia* (2n=6x=48). *Prunus* is economically important because of its diversified use and can be used as fruit (e.g., plums, peaches, apricots, cherries, and almonds), ornamentals, forestry or industrial purposes. The presence of large number of *Prunus* species increase the possibilities of interspecific hybridisation, and make the establishment of botanical classification quite complicated. Traditional taxonomy of *Prunus* has used morphology to estimate relationships among the species in question and to provide a taxonomy for such genus. Systematic classification in *Prunus* is still controversial. De Tournefort (1700) recognized six distinct genera within *Prunus* s.l. (sensu lato) based on fruit morphology: *Amygdalus* L., *Armeniaca* Miller, *Cerasus* Miller, *Laurocerasus* Duhamel, *Persica* Miller, and *Prunus* s.s. (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: sects. *Amygdalopsis*, *Amygdalus*, *Armeniaca*, *Cerasoides*, *Cerasus*, *Laurocerasus*, and *Prunus*. Koehne (1893) initially divided the genus *Prunus* into seven subgenera, but later (Koehne, 1911) recognized 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 Rehder, was composed of a large and diverse group of species and was later divided by Ingram (1948) into subgenera *Cerasus* and *Lithocerasus* Ingram. Nowadays, biochemical (isoenzyme) and molecular (DNA analysis) approaches allow more accurate estimate of genetic affinities and evolutionary relatedness among *Prunus* taxa that would permit to compare traditional classifications established by taxonomists. Several investigations were carried out to assess genetic variation among *Prunus* species using isozyme markers (Arulsekar et al., 1986; Mowrey

and Werner, 1990). Molecular markers as RFLPs, RAPDs and SSRs were also used for phylogenetic and genetic diversity studies among *Prunus* species (Kaneko et al., 1986; Casas et al., 1999; Rohrer et al., 2004).

Noncoding regions of the chloroplast DNA (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 (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapegue et al., 1997) 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 fragment (Demesure et al., 1996; El Mousadek and Petit, 1996; King and Ferris, 1998). This PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method, also named CAPS (Cleaved Amplified Polymorphic Sequences) (Konieczny and Ausubel, 1993), is a readily accessible laboratory technique which can evaluate large portions of chloroplast genome in numerous individuals in a short time and lower cost. This method has been used to analyse 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 favourable traits which appear in different species (Moreno, 2004). There are commercial *Prunus* rootstocks available on the market which are a result of uncontrolled interspecific pollinations. Moreover, the pedigree of most of the clones is unknown due to the lack of parental control and which is a major constraint for their use in breeding programmes. It is subsequently desirable to appropriately characterise the parentage of the rootstock material as a means to evaluate the success of the crossing.

In this study, a characterisation of *Prunus* species and rootstocks (interspecific hybrids) on the basis of cpDNA screening was carried out with the following objectives: (1) to analyse 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.

6-2. MATERIALS AND METHODS

6-2-1. Plant material

Sixty two accessions of various interspecific hybrids of the genus *Prunus* were obtained from the Krymsk Experimental Breeding Station of Russia (KEBS, Russia) including pedigree data (see Table 1). These accessions are representative of the genetic variability in the KEBS collection. Twenty two *Prunus* species were sampled from three important collections of *Prunus* in Europe: KEBS, Russia; Estación Experimental de Aula Dei-CSIC, Zaragoza, Spain; INRA (Institut Nationale de Recherche Agronomique) of Bordeaux and INRA of Avignon, France (Table 1). Fresh leaves were collected from plants, then frozen and stored at –80 ºC.

6-2-2. DNA extraction, amplification, and digestion

DNA was extracted from frozen leaves following the CTAB (cethyltrimethyl ammonium bromide) 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 by spectrophotometer. Extracted DNA was diluted to 5 ng/µL (working solution) and stored at -20 ºC until PCR reactions.

Initially, amplifications of cpDNA were done using five universal primer pairs (Table 6-2) described by Dumolin-Lapegue et al. (1997). PCR reactions were performed in a final volume of 40 µL containing 20 ng of template DNA, 0.2 mM of each of the four dNTPs, 0.2 µM 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 and M Labs S.A., Spain). Amplifications were carried out in a Gene Amp 2700 thermocycler (Applied Biosystems, Foster, CA) using the following temperature cycles: 1 cycle of 3 min at 95 ºC, 40 cycles of 1 min at 94 ºC, 45 s at 52 ºC to 62 ºC, and 2 min to 5 min 30 s at 72 ºC (annealing temperature and extension time for each primer pair are in Table 6-2). The last cycle was followed by a final incubation for 10 min at 72 ºC, and then the PCR products were stored at 4 ºC before analysis. Amplification of cpDNA products were checked by electrophoresis on 1% w/v agarose gels, stained with ethidium bromide, and visualised under UV light. Approximate amplified fragment sizes were estimated with a 1-kb ladder DNA marker (Invitrogen, Carlsbad, Calif.).

Table 6-1. *List of 84 accessions (interspecific hybrids and species) of Prunus studied. Classification into sections and subgenera is according to Rehder (1940). Haplotypes found in accessions are also included.*

Accession Number	Origin[a]	Genotype (♀ x ♂)	Section[b]	Subgenus[b]	Haplotype
PACE00-03	KEBS	[(***P. pumila*** *x P. armeniaca*) *x P. domestica*]	*Microcerasus*	*Cerasus*	H1
PACE00-08	KEBS	[(***P. pumila*** x *P. armeniaca*) x *P. domestica*] (4x)	*Microcerasus*	*Cerasus*	H1
PACE01-26	KEBS	(***P. besseyi*** x *P. salicina*) x (*P. cerasifera* x *P. persica*)	*Microcerasus*	*Cerasus*	H1
PACE01-34	KEBS	[(***P. besseyi*** x *P. armeniaca*) x *P. domestica*]	*Microcerasus*	*Cerasus*	H1
PACE01-46	KEBS	(***P. pumila*** x *P. armeniaca* x *P. domestica*)	*Microcerasus*	*Cerasus*	H1
PACE02-14	KEBS	[(***P. pumila*** x *P. salicina*) x (*P. cerasifera* x *P. persica*)]	*Microcerasus*	*Cerasus*	H1
PADAC01-47	KEBS	(***P. pumila*** x *P. armeniaca x P. domestica*)	*Microcerasus*	*Cerasus*	H1
Drujba	KEBS	***P. pumila*** x *P. armeniaca*	*Microcerasus*	*Cerasus*	H1
EVRICA	KEBS	[(***P. besseyi*** x *P. salicina*) x (*P. cerasifera* (Otletchniza))]	*Microcerasus*	*Cerasus*	H1
Krasnaia-lenta	KEBS	(***P. besseyi*** x *P. salicina*) x *P. cerasifera*) = Hiawatha x *P. cerasifera*	*Microcerasus*	*Cerasus*	H1
Krasni bordur	KEBS	[(***P. besseyi*** x *P. salicina*) x *P. cerasifera* (Pissardi)]	*Microcerasus*	*Cerasus*	H1
ST-N1	KEBS	***P. besseyi*** x *P. cerasifera*	*Microcerasus*	*Cerasus*	H1
ST-N2	KEBS	***P. besseyi*** x *P. cerasifera*	*Microcerasus*	*Cerasus*	H1
AD-3	EEAD	***P. besseyi*** L.H. Bailey	*Microcerasus*	*Cerasus*	H1
PACE01-35	KEBS	(***P. salicina*** x *P. besseyi*) x (*P. cerasifera* x *P. armeniaca*)	*Euprunus*	*Prunus*	H2
PACE02-05	KEBS	(***P. salicina*** x *P. cerasifera*) x (*P. cerasifera* x *P. persica*)	*Euprunus*	*Prunus*	H2
PACE02-06	KEBS	(***P. salicina*** x *P. cerasifera*) x (*P. armeniaca* x *P. persica*)	*Euprunus*	*Prunus*	H2
PACE02-07	KEBS	[***P. salicina*** x *P. cerasifera* (4x)] x [*P. cerasifera* x *P. persica* (4x)]	*Euprunus*	*Prunus*	H2
PACE02-08	KEBS	[(***P. salicina*** x *P. cerasifera*) x (*P. armeniaca* x *P. persica*)]	*Euprunus*	*Prunus*	H2
PACE02-21	KEBS	(***P. salicina*** (Santa Rosa) x P. cerasifera)(4x) x (*P. cerasifera* x *P. persica* (Kuban-86) (4x))]	*Euprunus*	*Prunus*	H2
AD-11	EEAD	***P. salicina*** Lindl.	*Euprunus*	*Prunus*	H2
PACE00-09	KEBS	***P. cerasifera*** x *P. persica* (4x)	*Euprunus*	*Prunus*	H3
PACE01-38	KEBS	***P. dasycarpa*** x *P. domestica*	*Armeniaca*	*Prunus*	H3
PACE01-39	KEBS	***P. dasycarpa*** x *P. spinosa* , F2	*Armeniaca*	*Prunus*	H3
PACE01-40	KEBS	***P. dasycarpa*** x *P. spinosa* , F2	*Armeniaca*	*Prunus*	H3
PACE01-41	KEBS	***P. dasycarpa*** x *P. spinosa* , F2	*Armeniaca*	*Prunus*	H3
PACE02-17	KEBS	***P. cerasifera*** x *P. persica* (nectarine)	*Euprunus*	*Prunus*	H3
PACE02-18	KEBS	***P. cerasifera*** x *P. persica* (Krymsk-86 (4x))	*Euprunus*	*Prunus*	H3
Krasnaia-Kubani	KEBS	***P. cerasifera*** x *P. persica* (Red leaf)	*Euprunus*	*Prunus*	H3
Kuban-87	KEBS	***P. cerasifera*** x *P. persica*	*Euprunus*	*Prunus*	H3
PACE01-13	KEBS	***P. fruticosa*** x *P. mahaleb*	*Eucerasus*	*Cerasus*	H4
PACG00-01	KEBS	***P. fruticosa*** x *P. pensilvanica*	*Eucerasus*	*Cerasus*	H4
LC-52	KEBS	***P. cerasus*** x *P. maackii*	*Eucerasus*	*Cerasus*	H4
VSL-1	KEBS	***P. fruticosa*** x *P. lannesiana*	*Eucerasus*	*Cerasus*	H4
VSL-2	KEBS	***P. fruticosa*** x *P. lannesiana*	*Eucerasus*	*Cerasus*	H4
AD-16	INRA-B	***P. fruticosa*** Pall.	*Eucerasus*	*Cerasus*	H4
AP-2	KEBS	***P. cerasifera*** x *P. persica* (nectarine)	*Euprunus*	*Prunus*	H5
Krymsk-1	KEBS	***P. cerasifera*** x *P. tomentosa*	*Euprunus*	*Prunus*	H5
PACE02-15	KEBS	***P. cerasifera*** x (*P. communis* x *P. bucharica*)	*Euprunus*	*Prunus*	H5
PACE01-42	KEBS	***P. kansuensis*** x *P. davidiana* x *P. persica*	*Euamygdalus*	*Amygdalus*	H6
PACE01-43	KEBS	***P. kansuensis*** x *P. davidiana* x *P. persica*	*Euamygdalus*	*Amygdalus*	H6
AD-19	INRA-A	***P. kansuensis*** Rehd.	*Euamygdalus*	*Amygdalus*	H6

***Table 6-1** (continued)*

Accession Number	Origin[a]	Genotype (♀ x ♂)	Section[b]	Subgenus[b]	Haplotype
PACE02-10	KEBS	***P. brigantiaca*** x *P. persica*	*Armeniaca*	*Prunus*	H7
PACE02-11	KEBS	***P. brigantiaca*** x *P. persica*	*Armeniaca*	*Prunus*	H7
AD-20	INRA-A	***P. brigantiaca*** Vill.	*Armeniaca*	*Prunus*	H7
PACE01-24	KEBS	***P. americana*** x *P. persica*	*Prunocerasus*	*Prunus*	H8
PACE02-09	KEBS	***P. americana*** x *P. persica*	*Prunocerasus*	*Prunus*	H8
PACE02-12	KEBS	***P. americana*** x *P. persica*	*Prunocerasus*	*Prunus*	H8
PACE00-02	KEBS	***P. tenella*** x *P. dulcis*	*Chameamygdalus*	*Amygdalus*	H9
PACG00-02	KEBS	***P. nana*** x *P. dulcis*	*Chameamygdalus*	*Amygdalus*	H9
AD-13	EEAD	***P. tenella*** Batsh	*Chameamygdalus*	*Amygdalus*	H9
PACE01-30	KEBS	***P. cerasifera*** x *P. bucharica*	*Euprunus*	*Prunus*	H10
PACE01-31	KEBS	***P. cerasifera*** x *P. bucharica*	*Euprunus*	*Prunus*	H10
PACE01-33	KEBS	***P. cerasifera*** x *P. kalmycovii*	*Euprunus*	*Prunus*	H10
PACE00-12	KEBS	[***P. spinosa*** x (*P. cerasifera* x *P. persica*) (Kuban-1)]	*Euprunus*	*Prunus*	H11
PACE01-27	KEBS	[***P. spinosa*** x (*P. cerasifera* x *P. persica*)]	*Euprunus*	*Prunus*	H11
PACE00-07	KEBS	***P. spinosa*** x *P. persica* (4x)	*Euprunus*	*Prunus*	H11
AD-2	EEAD	***P. armeniaca*** L.	*Armeniaca*	*Prunus*	H12
AD-9	EEAD	***P. persica*** (L.) Batsch	*Euamygdalus*	*Amygdalus*	H13
PACEOR-03	KEBS	[(***P. americana*** x *P. simonii*) x *P. spinosa*] x (*P. cerasifera* x *P. persica*)	*Prunocerasus*	*Prunus*	H14
Vesenia-plamiya	KEBS	[(***P. americana*** x *P. simonii*) x *P. cerasifera* (Pissardii)]	*Prunocerasus*	*Prunus*	H14
AD-18	INRA-B	***P. americana*** Marshall	*Prunocerasus*	*Prunus*	H14
PACE01-32	KEBS	***P. cerasifera*** x *P. bucharica*	*Euprunus*	*Prunus*	H15
PACE01-28	KEBS	[***P. cerasifera*** x (*P. amygdalus* x *P. bucharica*)]	*Euprunus*	*Prunus*	H16
PACE02-04	KEBS	***P. kansuensis*** x *P. persica* (Red leaf)	*Euamygdalus*	*Amygdalus*	H17
PACE01-37	KEBS	***P. cerasifera*** x *P. prostrata* , F2	*Euprunus*	*Prunus*	H18
PACE01-45	KEBS	***P. prostrata*** x *P. cerasifera* x *P. domestica*	*Microcerasus*	*Cerasus*	H18
PACE00-10	KEBS	***P. cerasifera*** x *P. bucharica*	*Euprunus*	*Prunus*	H19
AD-14	EEAD	(***P. besseyi*** x *P. salicina*) (Sapa)	*Microcerasus*	*Cerasus*	H20
PACE00-06	KEBS	[(***P. besseyi*** x *P. salicina*) (Sapa) x (*P. cerasifera* x *P. tomentosa*) (Krymsk-1)]	*Microcerasus*	*Cerasus*	H20
VSV-1	KEBS	***P. incana*** x *P. tomentosa*	*Microcerasus*	*Cerasus*	H20
AD-21	EEAD	***P. prostrata*** Labill.	*Microcerasus*	*Cerasus*	H21
AD-17	EEAD	***P. mahaleb*** (Dougl.) L.	*Mahaleb*	*Cerasus*	H22
AD-4	EEAD	***P. cerasifera*** Ehrh.	*Euprunus*	*Prunus*	H23
AD-8	EEAD	***P. insititia*** L.	*Euprunus*	*Prunus*	H24
L-2	KEBS	***P. lannesiana*** (Carr.) E.H. Wilson	*Pseudocerasus*	*Cerasus*	H25
AD-22	EEAD	***P. bucharica*** (Korsch.) Hand-Mazz.	*Euamygdalus*	*Amygdalus*	H26
AD-12	EEAD	***P. spinosa*** L.	*Euprunus*	*Prunus*	H27
AD-10	EEAD	***P. pumila*** L.	*Microcerasus*	*Cerasus*	H28
AD-7	EEAD	***P. incana*** (Pall.) Basch.	*Microcerasus*	*Cerasus*	H29
AD-15	INRA-B	***P. davidiana*** (Carriere) Franch.	*Euamygdalus*	*Amygdalus*	H30
AD-5	EEAD	***P. dasycarpa*** Ehrh.	*Armeniaca*	*Prunus*	H31
AD-6	EEAD	***P. domestica*** L.	*Euprunus*	*Prunus*	H32
AD-1	EEAD	***P. dulcis*** (Mill.) D.A. Webb.	*Euamygdalus*	*Amygdalus*	H33

[a] Collection origins: KEBS = Krymsk Experimental Breeding Station of Russia; EEAD = Estación Experimental de Aula Dei-CSIC, Zaragoza, Spain; INRA-A = Institut Nationale de Recherche Agronomique of Avignon, France; INRA-B = Institut Nationale de Recherche Agronomique of Bordeaux, France. [b] Section and subgenus given here correspond to species in bold type, representing female parent or maternal progenitor for the interspecific hybrid accessions.

Amplified fragments were digested with three restriction enzymes: *Alu*I, *Hinf*I, and *Taq*I (Invitrogen, Carlsbad, Calif.). The reaction mixture was incubated overnight at 37 ºC with three units of *Alu*I or *Hinf*I, or 1 h at 65 ºC with five units of *Taq*I. Restriction products were separated on 2.5% w/v agarose gels, run in a Tris-borate-EDTA buffer (1x) for 5 h at 3V/cm, then stained with ethidium bromide and visualized under UV light. Restriction fragment sizes were estimated with a 50-bp ladder marker (Amersham, Piscataway, N.J.).

6-2-3. Data analysis

The presence or absence of each restriction fragments in 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 the unweighted pair-group method with arithmetic averages (UPGMA). All the analyses were computed using the programs from the NTSYS-pc package, version 2.1 (Rohlf, 2000).

Phylogenetic relationships were recovered using a maximum parsimony approach based on the binary data matrix performed for the UPGMA cluster analysis. Searches for the most parsimonious trees were executed with PAUP* version 4.0 (Swofford, 1999). Parsimony analysis was carried out through an heuristic search with CLOSEST addition of samples, tree bisection reconnection (TBR) branch swapping algorithm, and the MULPARS option on Bootstrap support (Felsenstein, 1985) was estimated through 10000 replicates imposing the same conditions as for the heuristic search. The consistency index (CI) and the retention index (RI) were used to assess the amount of homoplasy and the synapomorphic value present in the data set, respectively.

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 in calculating the number of mutational differences between haplotypes, and then connect them by direct links having the smallest possible length (Prim, 1957).

Table 6-2. *Chloroplast DNA universal primer pairs used in this study. PCR conditions, size of amplified fragments and quality of amplification.*

Abrev. of cpDNA primers[a]	PCR conditions		Amplified fragment (bp)	Degree of amplification[b]
	Annealing temperature (°C)	Extension time		
DT	52	2 min 30 s	1,15	++
HK	62	3 min	1,7	++
K1K2	57	4 min	2,65	++
CD	57	5 min 30 s	3,8	+
VL	56	5 min 30 s	3,9	NA

[a] Abbreviations as described by Dumolin-Lapegue et al. (1997).

[b] ++: good amplification; +: weak amplification; NA: no amplification.

6-3. RESULTS

6-3-1. Chloroplast DNA restriction patterns

The polymorphism in noncoding regions of the chloroplast DNA was studied in 84 interspecific hybrids and species of the genus *Prunus*. Initially, five universal primer pairs of cpDNA were used for amplification. Thereafter, and given the good degree of amplification (Table 6-2), three primer pairs (DT, HK, K1K2) were chosen for this study. Estimated sizes of the fragments amplified by DT, HK, and K1K2 were 1,150 bp, 1,700 bp, and 2,650 bp, respectively. Total size of these fragments represent approximately 4% of the total chloroplast genome (considering that cpDNA size in most *Prunus* spp. is approx. 140 kpb; 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 (*Alu*I, *Hinf*I, and *Taq*I). From the initial nine primer pair-restriction enzyme combinations, eight (DT-*Alu*I, DT-*Hinf*I, DT-*Taq*I, HK-*Alu*I, HK-*Hinf*I, K1K2-*Alu*I, K1K2-*Hinf*I, K1K2-*Taq*I) gave polymorphic patterns (Table 6-3). The HK-*Taq*I combination showed a monomorphic pattern.

Table 6-3. *Major pattern and variant (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[a] (bp)
DT-(*Alu1*)1	675 (B) → 720 (A)
DT-(*Alu1*)2	475 (B) → 490 (A), 460 (C), 250 + 225 (D)
DT-(*Hin* f1)1	305 (B) → 315 (A), 175 + 140 (C)
DT-(*Hin* f1)2	260 (C) → 275 (B), 305 (A)
DT-(*Hin* f1)3	255 (A) → 250 (B)
DT-(*Taq1*)1	550 (B) → 555 (A), 540 (C)
DT-(*Taq1*)2	395 (C) → 400 (B), 375 (D), 430 (A)
DT-(*Taq1*)3	140 (B) → 150 (A)
HK-(*Alu1*)1	560 (B) → 570 (A)
HK-(*Alu1*)2	385 (D) → 405 (B), 375 (E), 350 (F), 415 (A), 400 (C), 310 (G)
HK-(*Alu1*)3	65 + 45 (B) → 110 (A)
HK-(*Hin* f1)1	690 (C) → 680 (D), 660 (E), 640 (F), 710 (A), 700 (B)
HK-(*Hin* f1)2	240 (C) → 245 (B), 250 (A)
K1K2-(*Alu1*)1	425 (A) → 420 (B)
K1K2-(*Alu1*)2	330 + 130 (B) → 320 + 130 (D), 460 (A), 325 + 130 (C)
K1K2-(*Hin* f1)1	230 + 255 (B) → 485(A)
K1K2-(*Hin* f1)2	380 (A) → 370 (B)
K1K2-(*Hin* f1)3	340 (C) → 450 (A), 360 (B)
K1K2-(*Hin* f1)4	190 (B) → 200 (A)
K1K2-(*Taq1*)1	700 (A) → 670 (B)
K1K2-(*Taq1*)2	650 (C) → 700 (A), 655 (B), 600 (D)
K1K2-(*Taq1*)3	340 (A) → 335 (B), 270 (C)
K1K2-(*Taq1*)4	265 (A) → 250 (D), 260 (B), 255 (C)

[a] A, B, C, D, E, F, and G represent different polymorphisms in each polymorphic site.

These eight primer-enzyme combinations generated 23 polymorphic sites with 41 length mutations (indel mutations) and seven point mutations. Three primer-enzyme combinations (DT-*Alu*I, HK-*Hinf*I, and K1K2-*Alu*I) showed two polymorphic sites, while DT-*Hin*fI, DT-*Taq*I, and HK-*Alu*I presented three, and K1K2-*Hinf*I and K1K2-*Taq*I showed four polymorphic sites (Table 6-3).

Of the 23 polymorphic sites, 13 showed three or more patterns and the most polymorphic were HK-(*Alu*I)2 and HK-(*Hinf*I)1 with 7 and 6 different patterns,

respectively. The rest of the polymorphic sites presented only two different restriction patterns (Table 6-3).

The restriction patterns obtained in the combination K1K2-*Hinf*I in 11 interspecific hybrids are shown in Figure 6-1. The first interspecific hybrid shows a different restriction pattern from the rest with the presence of a fragment of approx. 485 bp, which is substituted by two fragments of 230 bp and 255 bp in the rest of the samples, caused by a point mutation (Table 6-3). Also, most of the interspecific hybrids show a fragment of approx. 340 bp except the fourth and the fifth ones which 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.

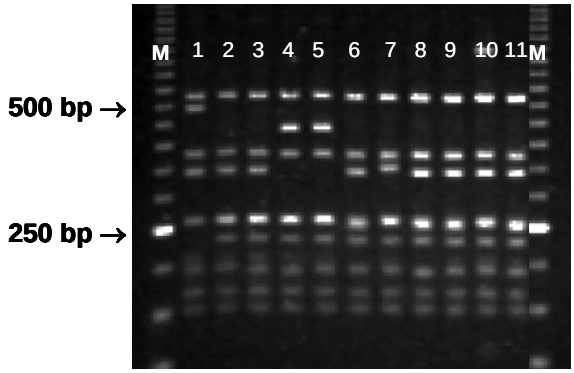

Figure 6-1. *Restriction patterns obtained on agarose gel with the primer pair-restriction enzyme combination K1K2-HinfI in 11 interspecific hybrids of Prunus. M: molecular size marker (50 base pair ladder, Amersham, Piscataway, N.J.)*

6-3-2. Chloroplast DNA haplotypes

The combination of all the mutations resulted in 33 cpDNA haplotypes (described in Table 6-4), which represent the 84 accessions studied as are shown in Table 6-1. 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* specie, while the remaining six (H1, H3, H4, H9, H18 and H20) were shared by two different species (Table 6-1). On the other hand, fifteen from the 24 species analysed (directly or through their interspecific hybrids) presented only one haplotype, while eight species (*P. americana*, *P. besseyi*, *P. dasycarpa*,

Table 6-4. *Description of 33 cpDNA haplotypes showing mutational differences in each polymorphic site, revealed by the eight combinations primer-restriction enzyme used in this study.*

	Haplotype																														
Polymorphic site	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-(*Alu* 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	A	B	B	B
DT-(*Alu* I)2	B	B	B	A	B	B	A	B	B	B	C	B	B	B	B	B	B	D	B	B	D	A	B	B	A	B	B	B	B	B	B	B	B
DT-(*Hinf* 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	C	B	B	B	A	B	B	B	B
DT-(*Hinf* I)2	C	C	C	B	C	C	C	C	C	C	C	C	B	C	C	C	B	C	C	C	C	C	C	C	C	C	C	C	C	A	C	C	C
DT-(*Hinf* I)3	A	A	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
DT-(*Taq* 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	A	B	B	B	B	B	B	B	B
DT-(*Taq* 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	B	A	C	C	C
DT-(*Taq* 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	A	B	B	B	B	B	B
HK-(*Alu* 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	A	A	A	B	B
HK-(*Alu* I)2	B	D	D	D	D	D	D	E	D	D	B	B	D	E	D	D	D	D	A	B	A	C	D	G	B	D	F	D	D	D	D	G	D
HK-(*Alu* 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
HK-(*Hinf* I)1	C	E	D	C	D	D	D	E	E	C	D	C	C	E	C	C	D	D	C	C	A	C	D	C	B	D	F	C	E	E	D	D	D
HK-(*Hinf* I)2	B	B	C	C	C	C	C	A	C	C	C	C	C	C	C	C	C	A	C	C	B	C	C	C	C	C	C	B	C	A	C	C	C
K1K2-(*Alu* 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	B	A	A	A	A	A	A	A	A
K1K2-(*Alu* I)2	B	B	D	B	B	B	B	A	B	B	B	B	B	A	D	D	B	B	B	B	B	B	B	B	B	C	B	B	B	B	B	B	C
K1K2-(*Hinf* I)1	B	B	A	B	B	B	A	B	B	A	A	B	B	B	A	A	B	B	B	B	B	B	A	A	B	B	A	B	B	B	A	A	B
K1K2-(*Hinf* I)2	A	A	B	A	A	A	A	A	A	A	A	A	A	A	B	B	A	A	A	A	A	A	A	A	A	B	A	A	A	A	B	A	B
K1K2-(*Hinf* I)3	C	C	C	C	C	A	C	C	B	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-(*Hinf* 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
K1K2-(*Taq* 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
K1K2-(*Taq* I)2	C	C	A	C	C	C	C	C	D	A	B	C	C	A	A	A	C	C	C	C	C	C	A	A	C	C	C	C	C	C	A	A	C
K1K2-(*Taq* 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	C	A	A	A	A	A	A
K1K2-(*Taq* 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	C	A	A	A	A	A	A	A

A, B, C, D, E, F and G: different patterns of each polymorphic site (see Table 6-3 for code).

P. kansuensis, *P. incana*, *P. prostrata*, *P. pumila,* and *P. spinosa*) presented two different haplotypes. *P. cerasifera* is the unique species of this study which showed eight different haplotypes (Table 6-1).

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 6-1). Other hybrid accessions with *P. besseyi* as female parent shared the haplotype H20 with an interspecific hybrid with *P. incana* as maternal progenitor (Table 6-1).

The haplotype H2 was only shared by seven accessions, six were interspecific hybrids showing *P. salicina* as female parent and the last one represented the species *P. salicina* itself. Similar results were found in haplotype H7, which were only detected in two interspecific hybrids with *P. brigantiaca* as female parent and the species *P. brigantiaca* itself. Moreover, haplotypes H6 was detected in two interspecific hybrids with *P. kansuensis* as female parent and the species *P. kansuensis* itself, but this species was also represented by another haplotype H17 (Table 6-1). The *P. americana* accession and two interspecific hybrid with *P. americana* as female parent shared the same haplotype H14. This species also showed haplotype H8 (Table 6-1).

All interspecific hybrid accessions with *P. dasycarpa* as female parent shared the same haplotype H3 with some hybrid accessions presenting *P. cerasifera* as female parent. The *P. dasycarpa* species also showed the haplotype H31. Moreover, *P. cerasifera* presented other six haplotypes (H5, H10, H15, H16, H19 and H23) and shared the haplotype H18 with *P. prostrata* (Table 6-1). The haplotype H4 was common to all the accessions presenting *P. fruticosa* or *P. cerasus* as female parent, and to the species *P. fruticosa* itself. *Prunus tenella* accession and two interspecific hybrids with *P. tenella* or *P. nana* as female parent were included under the same haplotype H9.

6-3-3. Chloroplast DNA diversity and phylogenetic relationship 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 (Figure 6-2).

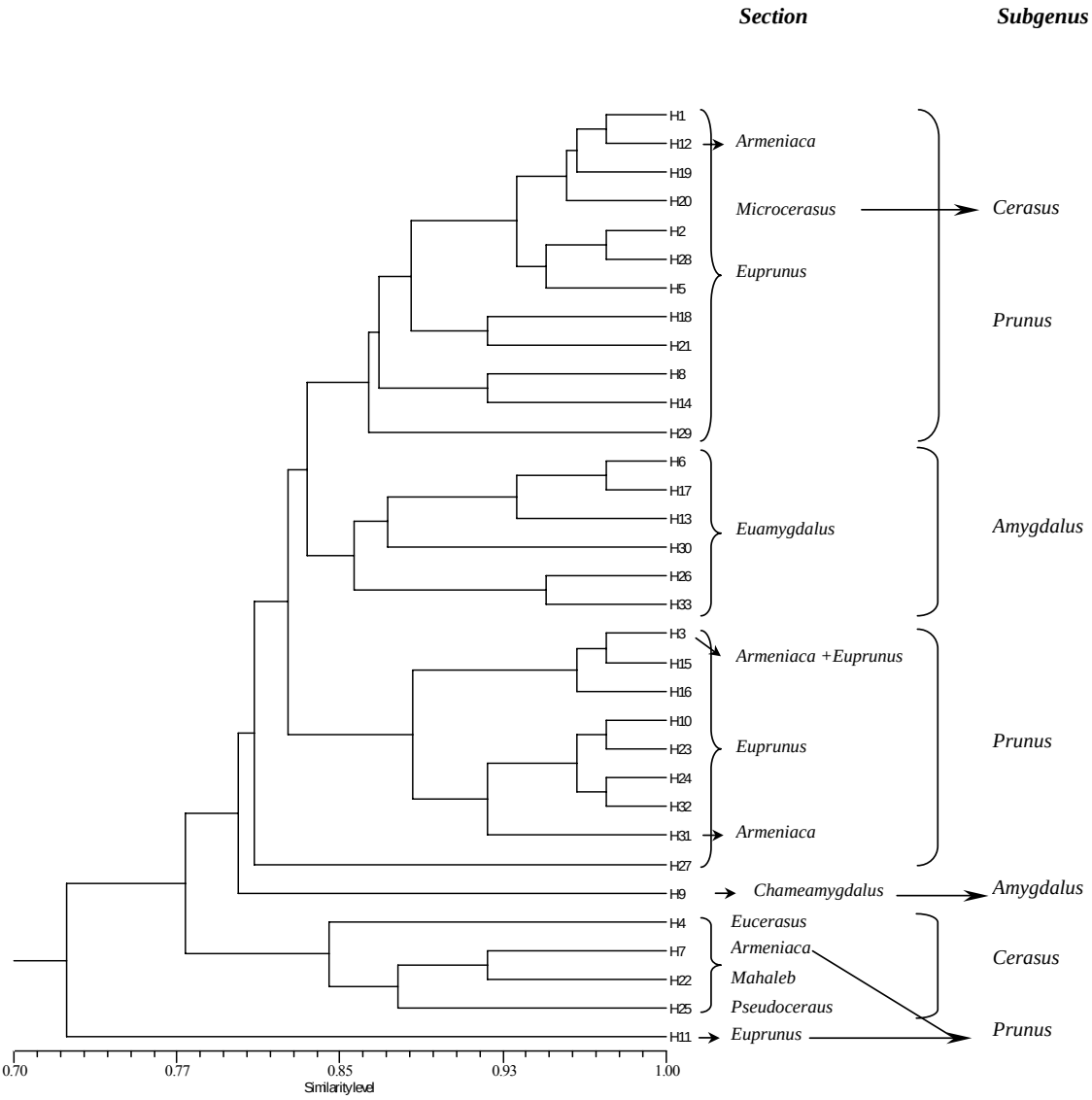

Figure 6-2. *Dendrogram of 33 Prunus cpDNA haplotypes (H1 to H33) generated by UPGMA clustering analysis using the simple matching coefficient. Classification into subgenera and sections is according to Rehder (1940).*

The first main group comprised haplotypes of *Prunus* species belonging to the subgenus *Prunus* (= *Prunophora*), *Amygdalus* and *Cerasus* sect. *Microcerasus*. 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* sect. *Eucerasus* (H4), sect. *Mahaleb* (H22), and sect. *Pseudocerasus* (H25), and the fourth haplotype (H7) was present in *P. brigantiaca* species, member of subgenus *Prunus* sect. *Armeniaca*. Two of the three haplotypes clustered out of groups was 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 sub-groups were observed (Figure 6-2). A close relationship was detected among haplotypes included in the first sub-group, representing species of the subgenus *Prunus* sections *Armeniaca*, *Euprunus*, and *Prunocerasus*, and species of the subgenus *Cerasus* sect. *Microcerasus* (H1, H20, H21, H28, and H29; see Table 6-1). Haplotypes representing species of the subgenus *Amygdalus* sect. *Euamygdalus* were clustered together under the second sub-group (Figure 6-2). The third sub-group, clustered at a similarity value of 0.82 from the previous two, contained haplotypes which mostly represented species of subgenus *Prunus* sect. *Euprunus*. Haplotypes representing species of sect. *Armeniaca* (H3 and H31) were also included in this sub-group.

Maximum parsimony analysis

Maximum parsimony analysis of cpDNA haplotypes generated 56 most parsimonious trees (MPT) [Length (L) = 139 steps, Consistency index (CI) = 0.51, Retention index (RI) = 0.65]. The strict consensus tree showed two main clades (Figure 6-3). The first one included haplotypes representing species of subgenera *Amygdalus*, *Cerasus*, and *Prunus*; though interspecific relationships were unresolved. Haplotypes representing species of the subgenus *Amygdalus* sect. *Euamygdalus* emerged as monophyletic group. Within this *Amygdalus* group, the sister taxa *P. bucharica* (H26) and *P. dulcis* (H33) showed moderate bootstrap support (76%). Four haplotypes of subgenus *Cerasus* (H4, H25, H22, H29) plus an haplotype (H7) of *P. brigantiaca*, a species traditionally classified in *Prunus* sect. *Armeniaca*, joined into the same group. Other haplotypes of subgenus *Cerasus* (H1, H20, H28) were related to this group but joined in a polytomy with other haplotypes representing species of the subgenus *Prunus*

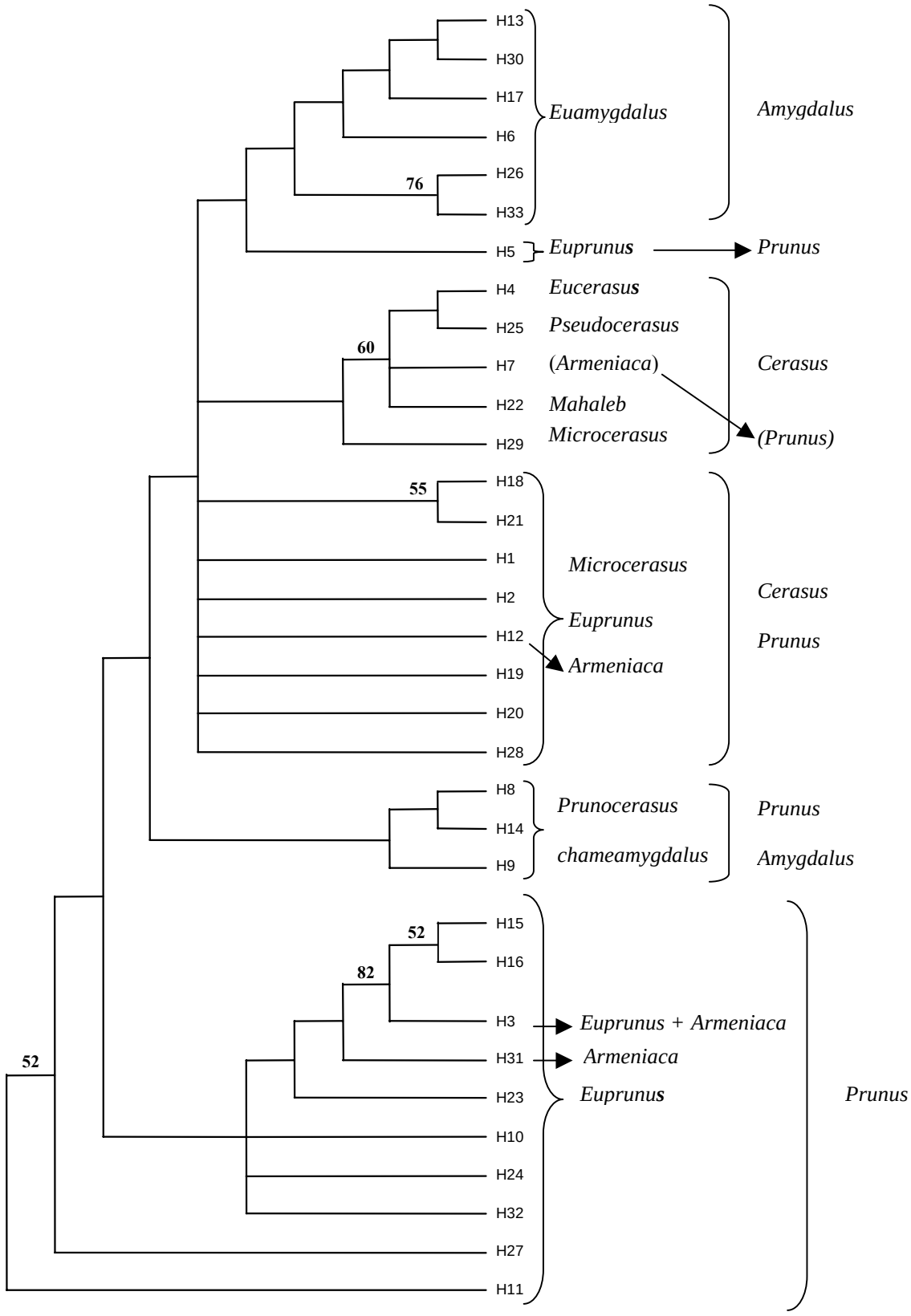

Figure 6-3. *Strict consensus of 56 most parsimonious trees of 33 Prunus cpDNA haplotypes (L = 139, CI = 0.51, RI = 0.65). Bootstrap percentages, when greater than 50%, are shown above the branches. Classification into subgenera and sections is according to Rehder (1940).*

(H21, H2, H12 and H19). The haplotypes H18 (*P. prostrata* and *P. cerasifera*) and H21 (*P. prostrata*) were resolved as sister haplotypes although with low support (55%) (Figure 6-3).

The second main clade included haplotypes representing *Prunus* species of subgenus *Prunus* sect. *Euprunus*, except those of *P. dasycarpa* (H3 and H31) that belong to sect. *Armeniaca* (Figure 6-3). Haplotypes H15, H16 and H3, representing *P. cerasifera* and *P. dasycarpa* species, joined into a strong supported group with high support (82%). Similarly to the UPGMA phenogram, haplotypes H27 and H11 (from *P. spinosa* species) were also clustered out of clades in the consensus tree (Figure 6-3).

Minimum-length spanning tree analysis

The relationships among the 33 cpDNA haplotypes were also presented in a minimum-length spanning tree (MST) showed in Figure 6-4. 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 *Prunus* sect. *Euprunus*, and sect. *Armeniaca* for *P. dasycarpa* (H3, H31) species. The cluster linked to H5 was composed of haplotypes representing species of subgenus *Amygdalus*, *Cerasus* and *Prunus*.

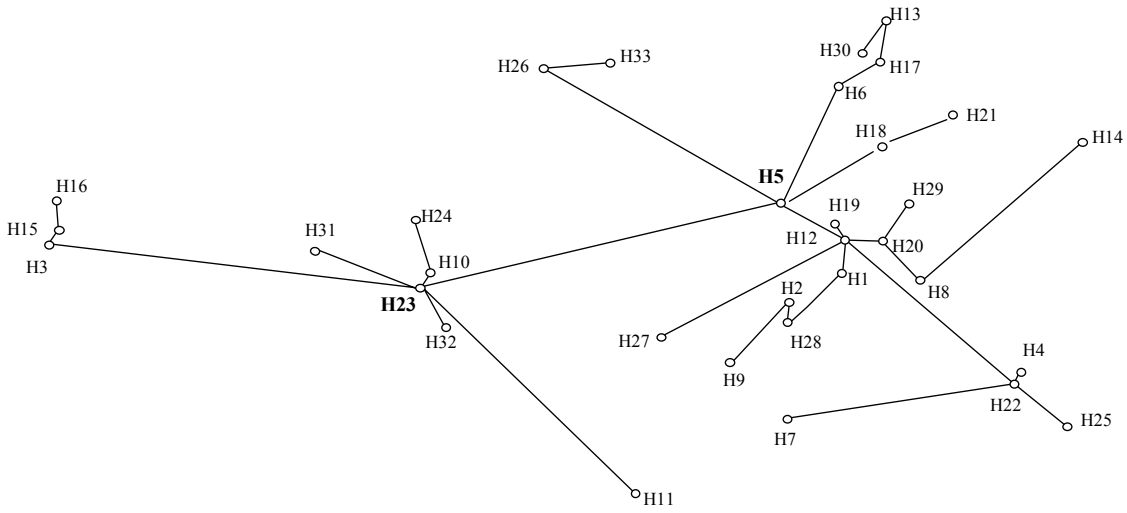

Figure 6-4. *The minimum-length spanning tree of 33 cpDNA haplotypes from the 84 Prunus accessions studied.*

6-4. DISCUSSION

6-4-1. Chloroplast DNA haplotypes: inheritance and relationships

The 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 6-1), which is very interesting for 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.

The haplotype H2 was shared by the species *P. salicina* and six complex interspecific hybrid of *Prunus* which have *P. salicina* as maternal progenitor (Table 6-1). 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 6-1).

These results seem to agree with the maternal inheritance of cpDNA in *Prunus* and, in some cases, permit to confirm the identity of the maternal progenitor. However, some interspecific hybrids showed different haplotypes from the obtained maternal ones.

This fact was observed when the *Prunus* species analysed directly or through their interspecific hybrids presented two or plus 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 of this study which showed eight different haplotypes, probably due to the large number of *P. cerasifera* accessions (16) analysed (directly or through their interspecific hybrids) (Table 6-1). The mentioned discordances could be due to the existence of intraspecific cpDNA variation, which has been previously reported in *Prunus* species as *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 need to analyse more accessions of these *Prunus* species to know better their intraspecific cpDNA diversity.

Several pairs of *Prunus* species shared the same cpDNA haplotypes (H1, H3, H4, H9, H18, and H20; Table 6-1). The predominant haplotype H1 was presented in *P. besseyi* species accession and 13 hybrids of *Prunus* having *P. pumila* or *P. besseyi* as female parent (Table 6-1). Assuming that cpDNA is maternally inherited in *Prunus*, the obtained result provided 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* sect. *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 could indicate that exist 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* x *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* could be the maternal progenitor of *P. dasycarpa*.

The 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* sect. *Eucerasus.* In addition, sour cherry (*P. cerasus*) is considered to be the product of interspecific hybridisation of sweet cherry (*P. avium*) and ground cherry (*P. fruticosa*) (Olden and Nymbom, 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*.

The haplotype H9 was shared by three accessions, two are hybrids having *P. tenella* or *P. nana* as female parent and one represent the species *P. tenella* (Table 6-1). According to Rehder (1940), *P. tenella* and *P. nana* are synonyms, which can explain their cpDNA pattern similarity.

6-4-2. Chloroplast DNA diversity and phylogenetic 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, a strict consensus tree and a minimum-length spanning tree. Comparison of the cpDNA phenogram and cladogram with the taxonomic classification of *Prunus* based on morphological data (Rehder, 1940) shows similarities but also several apparent discrepancies.

In the UPGMA phenogram (Figure 6-2), the first main group contained all haplotypes representing species of subgenus *Cerasus* sect. *Microcerasus*, which clustered with haplotypes representing species of subgenera *Prunus* 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), noted that sect. *Microcerasus* does not show any relationship to the rest of the subgenus *Cerasus*. Likewise, Katayama and Uematsu (2005), also found that *Amygdalus*, *Prunus*, and *Cerasus* sect. *Microcerasus* formed a major group in the UPGMA tree performed using RFLP data from chloroplast DNA. Moreover, the parsimony-based strict consensus tree (Figure 6-

3) showed most sect. *Microcerasus* haplotypes (H1, H18, H20, and H28) joined into a polytomic clade and separated from the *Cerasus* group (H4, H22, H25, H29), although all of them belonged to the first main clade. Similar relation among haplotypes representing *Microcerasus* species and haplotypes representing the rest of species of the subgenus *Cerasus* was also showed in the MST analysis (Figure 6-4).

The first main group in the UPGMA phenogram was composed of 3 sub-groups (Figure 6-2). The first sub-group included species of sect. *Microcerasus* and species of subgenus *Prunus*. This sub-group 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 apricot, *P. armeniaca* (H12). Ramming 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 sect. *Microcerasus* was not monophyletic and most of its species might be more closely related to subgen. *Prunus* than to subgen. *Cerasus*. More research is required to find the closest relatives belonging to *Microcerasus* species.

Haplotypes representing species of the subgenus *Amygdalus* sect. *Euamygdalus* were grouped together in the second sub-group (Figure 6-2). The *P. kansuensis, P. persica*, and *P. davidiana* peach species examined in this study, and represented by haplotypes H6, H17, H13 and H30 (Table 6-1), clustered together at a high similarity value (0.86). The *P. bucharica* (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 sect. *Euamygdalus* were often classified as either almond-like or peach-like plants (Scorza and Okie, 1990), which is well supported by our findings. The parsimony-based strict consensus tree (Figure 6-3) and the MST (Figure 6-4) also showed close relationships among *Amygdalus* haplotypes (sect. *Euamygdalus*) and were in agreement with the UPGMA clustering. Nevertheless, *P. tenella* member of subgenus *Amygdalus* sect. *Chamaeamygdalus*, represented by haplotype H9, appears out of the *Amygdalus* group (Figures 6-2, 6-3, and 6-4). This result indicates that the cpDNA of *P. tenella* can be highly divergent from the other *Amygdalus* species. Kester et al. (1990) reported that sect. *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*) (Figure 6-2), was also reported by Casas et al. (1999) and Bouhadida et al. (2005) analysing the genetic diversity of *Prunus* rootstocks with RAPD and SSR markers,

respectively. These two species were closely related to *P. cerasifera*, represented by H10 and H23 (Figures 6-2, 6-3, and 6-4). *P. 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 probably *P. domestica* 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, the strict consensus tree and the MST (Figures 6-2, 6-3, and 6-4), haplotypes H11 and H27 representing the *P. spinosa* plum specie, appear separated of haplotypes representing other plum species. These results agree with studies that showed the high cpDNA diversity in *P. spinosa* (Mohanty et al. 2000; 2002).

Prunus fruticosa and *P. cerasus* species (haplotype H4) were grouped together with *P. brigantiaca* (H7), *P. mahaleb* (H22), and *P. lannesiana* (H25) (Figures 6-2, 6-3, and 6-4). 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. brigantiaca*, which belong to subgenus *Prunus* sect. *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*, 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. brigantiaca* (H7) within this group obscured its relationship with respect to other apricots. Bortiri et al. (2001) also reported that *P. brigantiaca* 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. brigantiaca* within the genus *Prunus* and to reach at 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 analyse the phylogenetic relationships among

species and cultivars of *Prunus*. This study contributes to understand the maternal inheritance of cpDNA in *Prunus*, and permits to confirm the maternal progenitors of different hybrids of this genus. The maternal inheritance of cpDNA and the phylogenetic 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*.

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CAPÍTULO 7

Discusión general

Los marcadores moleculares de tipo microsatélites (SSRs) juegan un papel muy importante en la caracterización, identificación y análisis de la diversidad genética en melocotonero.

Los marcadores SSRs tienen un elevado nivel de polimorfismo, son codominantes, y fáciles de detectar mediante la PCR. Por otro lado, una vez diseñados los cebadores, la amplificación de los microsatélites es sencilla, reproducible y de fácil automatización. La validez de los SSRs en estudios genéticos de melocotonero se ha demostrado por muchos equipos de trabajo (Cipriani et al., 1999; Testolin et al., 2000; Sosinski et al., 2000; Dirlewanguer et al., 2002; Aranzana et al., 2002; 2003). Estos marcadores tienen mayor eficiencia en comparación con otros marcadores moleculares como los isoenzímas (Messeguer et al., 1987), los RFLPs (Rajapakse et al., 1995) o los RAPDs (Warburton y Bliss, 1996).

En esta tesis se han utilizado un conjunto de microsatellites desarrollados en su mayoría en melocotonero (Testolin et al., 2000; Sosinski et al., 2000; Dirlewanguer et al., 2002; Aranzana et al., 2002) y en cerezo (Downey e Iezzoni, 2000; Joobeur et al., 2000), para la caracterización y el análisis de la diversidad genética: en 30 variedades locales de melocotonero procedentes de Aragón (Capítulo 3); 94 variedades locales de distintas regiones españolas y variedades comerciales de melocotonero (Capítulo 4); y en 44 patrones de distintas especies e híbridos interespecíficos de *Prunus* (Capítulo 5). Por otro lado, se analizó el DNA cloroplástico de 84 accesiones (híbridos inter-específicos y especies *Prunus*) mediante la técnica de PCR-RFLP, con el objetivo de confirmar el progenitor materno en el pedigrí de los híbridos ínterespecíficos y detectar las relaciones genéticas y filogenéticas entre las especies *Prunus* estudiadas (Capítulo 6).

7.1. ESTUDIO GENÉTICO CON SSRs EN CULTIVARES DE GERMOPLASMA LOCAL DE MELOCOTONERO

Se estudiaron 30 cultivares tradicionales de melocotonero originarios de Aragón, con el fin de caracterizar la variedad ‘Miraflores’ que tiene un gran interés económico (Moreno y Casas, 2002). Esta variedad tiene un pedigrí desconocido y se comparó con otras variedades locales para buscar sus supuestos parentales. Estos cultivares se estudiaron con 20 SSRs previamente seleccionados por ser polimórficos en melocotonero.

Las 20 parejas de cebadores amplificaron un total de 46 alelos con una media de 2,3 alelos por locus. Otros trabajos en melocotonero detectaron un mayor número medio de alelos por locus [(4,5) Testolin et al. (2000); (3,2) Aranzana et al. (2002); (4,2) Dirlewanger et al. (2002)]. El valor medio de la heterocigosidad esperada (He) y el valor medio del poder de discriminación (PD) fueron también más bajos que los valores observados por los mismos autores. El bajo nivel mostrado en los parámetros de variabilidad de los SSRs puede ser debido, en este caso, a la baja variabilidad genética entre los 30 cultivares estudiados, ya que 19 de ellos son posibles clones de la misma variedad 'Miraflores' y 14 de ellos mostraron el mismo perfil molecular con todos los SSRs utilizados. Por otro lado, el resto de los cultivares incluidos en este estudio son variedades tradicionales cultivadas en la misma región que los 'Miraflores'. Tres de ellas ('Tardío del Pilar', 'Amarillo de Septiembre' y 'Tipo Campiel') muestran características del fruto similares a los clones de 'Miraflores' y se encuentran a unas distancias genéticas muy cercanas, lo que sugiere que podrían tener la misma base genética e incluso formar parte de su pedigrí. El resto de los cultivares tradicionales se encuentran más alejados.

En esta tesis, se estudiaron también 94 cultivares de melocotonero, nectarina y paraguayo, con características morfológicas y fenológicas diferentes. La mayoría de los cultivares (64) pertenecen al germoplasma local español de la colección nacional de referencia, en su mayoría con carne amarilla dura y adherente al hueso. El resto son variedades comerciales que provienen en su mayoría de programas de mejora modernos. Los 94 cultivares se analizaron mediante 15 parejas de cebadores seleccionados por su mayor nivel de polimorfismo. Los 15 SSRs permitieron la identificación de todas las variedades estudiadas, es decir, cada variedad mostró un genotipo único. Las 15 parejas de cebadores detectaron un total de 101 alelos con un valor medio de 6,73 alelos por locus. Este valor medio es mayor que el observado en los 30 cultivares locales aragoneses caracterizados con 20 SSRs (Capítulo 3) y es también mayor que los valores medios encontrados por otros autores (Testolin et al., 2000; Aranzana et al., 2002; y Dirlewanger et al., 2002). El alto número de alelos observados en este trabajo puede ser explicado por el elevado número de variedades y por la utilización de otros microsatélites que son más polimórficos.

Del total de alelos amplificados, 92 están presentes en los 79 cultivares de melocotonero de carne dura, 59 en los ocho cultivares de carne blanda, y 44 en los siete de nectarina. Cuarenta y un alelos fueron específicos de los cultivares de melocotonero

de carne dura, mientras que las nectarinas y los melocotones de carne blanda mostraron un sólo alelo específico para cada grupo. El elevado número de alelos (41) específicos del grupo de melocotón de carne dura demuestra la alta diversidad genética encontrada en este grupo, con respecto a las nectarinas y los melocotones de carne blanda. Las distancias genéticas fueron mayores al comparar cultivares pertenecientes a diferentes grupos que entre los cultivares de un mismo grupo. Las mayores distancias se observaron al comparar cultivares de carne dura con cultivares de carne blanda, y principalmente entre cultivares españoles y el resto. Asimismo, Aranzana et al. (2003) observaron que el grupo de los melocotoneros de carne dura era el más diverso con 18 alelos específicos, en comparación con el grupo de las nectarinas y el de los melocotones de carne blanda, que mostraron 16 y 8 alelos específicos, respectivamente. Messeguer et al. (1987) y Aranzana et al. (2002) observaron también una mayor diversidad genética en los cultivares de carne dura españoles.

En el presente trabajo, el valor medio de la heterocigosidad esperada (0,57) y el valor medio del poder de discriminación (0,66), están de acuerdo con los valores encontrados por Aranzana et al. (2003). Estos resultados soportan la validez de los marcadores microsatélites para mostrar la variabilidad genética existente en melocotonero.

El valor medio del índice de fijación (F = 0.58) demuestra, según Hartl y Clark (1997), una gran diferencia genética dentro de los cultivares de melocotonero estudiados. Al mismo tiempo, este valor muestra un exceso de homozigosidad (Murray, 1996). Esto puede ser debido a dos factores principales: primero, la presencia de alelos nulos que llevan a una falsa observación del exceso de homocigosidad; segundo, las variedades estudiadas de origen español son selecciones a partir de poblaciones reproducidas por semilla durante generaciones, lo que dada la naturaleza esencialmente autógama del melocotonero, conduciría a un aumento de la homocigosidad, tal y como se ha observado en nuestros resultados. El melocotonero es efectivamente una especie autocompatible y se autopoliniza en condiciones naturales. La polinización cruzada en esta especie podría ser menor del 5% de los casos (Fogle, 1977), lo que podría explicar la desviación del equilibrio de Hardy-Weinberg.

Por otro lado, la gran diferencia genética detectada entre los cultivares estudiados de melocotonero puede ser explicada por el bajo nivel de intercambio de genes (*gene flow*) dentro de esta especie y en las diferentes áreas geográficas de cultivo de las variedades estudiadas. Observaciones similares se han mencionado para albaricoquero por Maghuly et al. (2005).

Los resultados obtenidos indican que dentro de los cultivares de carne dura existe una fuente importante de variación, con caracteres de interés para los programas de mejora en melocotonero, ya que los cultivares comerciales, sobre todo los de carne blanda, presentan una base genética muy estrecha, debida al uso de un reducido número de parentales en los cruzamientos (Scorza et al., 1985).

Los mejoradores genéticos son conscientes de la necesidad de utilizar nuevas fuentes de germoplasma para poder alcanzar nuevos retos en el desarrollo de variedades de melocotonero (Werner y Okie, 1998). Por ello, la caracterización del germoplasma local en España constituye una etapa muy importante para la conservación y el eficiente manejo de su riqueza genética. La utilización sobre todo de cultivares de carne dura españoles, en los programas de mejora, podría aportar una mayor variabilidad a las nuevas variedades en selección.

La identificación total de los 94 cultivares de melocotonero, nectarina y paraguayo se pudo realizar con sólo 8 de los 15 SSRs utilizados. Es, por tanto, muy conveniente la elección de los marcadores más polimórficos y que, a su vez, se encuentren suficientemente distantes entre sí en los cromosomas del melocotonero (Aranzana et al., 2003).

El uso de geles de poliacrilamida para la separación electroforética de los fragmentos amplificados mediante la PCR resultó muy eficiente en este trabajo, dada la mayor resolución de los mismos al comparar con los geles de agarosa. Wünsch et al. (2005) examinaron 34 de los cultivares españoles de melocotonero incluidos en este trabajo con 10 SSRs, en geles de agarosa, no llegando a diferenciar algunos de ellos. Por el contrario, en este trabajo, hemos encontrado perfiles genotípicos únicos para cada uno de dichos cultivares. Estos resultados se explican por el uso, en nuestro caso, de marcadores más polimórficos, y por la utilización de una técnica de electroforesis más resolutiva.

7.2. ESTUDIO GENÉTICO DE PATRONES DE *Prunus* CON SSRs

El control y la utilización eficiente de los patrones *Prunus* en los programas de mejora requieren una caracterización previa del material. El Capítulo 5 de esta tesis describe la utilización de 20 marcadores microsatélites, desarrollados en melocotonero (Aranzana et al., 2002; Dirlewanger et al., 2002; Testolin et al., 2000), para la caracterización molecular de 44 patrones de *Prunus*, clasificados en tres grupos: los híbridos de melocotonero (*P. persica* x *P. davidiana*) y los híbridos almendro x melocotonero (*P. dulcis* x *P. persica*); los ciruelos de crecimiento rápido (patrones Mirobolán y Mariana) y los ciruelos de crecimiento lento (patrones *P. insititia* y *P. domestica*). Los marcadores SSRs se utilizaron, en este caso, por sus buenas características de polimorfismo, y por ser marcadores transferibles entre especies cercanas, como han demostrado distintos autores (Cipriani et al., 1999; Downey e Iezzoni, 2000; Sosinski et al., 2000).

De los 20 cebadores utilizados, 13 generaron buenas amplificaciones en todos los patrones *Prunus* estudiados, confirmando la transferibilidad de los SSRs entre especies cercanas. Solo tres cebadores (BPPCT001, CPPCT022 y UDP98-407) de los 13 utilizados permitieron la identificación total de los 44 patrones. Los marcadores seleccionados en este trabajo pueden ser utilizados en trabajos futuros de identificación de patrones *Prunus*.

En el primer grupo de patrones (híbridos con base genética de melocotonero y almendro), amplificaron 5 SSRs además de los 13 ya mencionados. Este hecho puede ser explicado porque los 20 marcadores SSR fueron previamente desarrollados en melocotonero, como se ha mencionado anteriormente.

Los dos grupos de ciruelos (ciruelos de crecimiento rápido y de crecimiento lento) mostraron el número más alto de alelos en común, lo que se explica por la estrecha relación genética entre los clones de cada grupo y por el hecho de que estos dos grupos pertenecen al mismo subgénero *Prunophora*. Por otro lado, las especies *P. domestica* y *P. insititia* (grupo de los ciruelos de crecimiento lento) podrían proceder del cruzamiento entre la especie diploide Mirobolán (*P. cerasifera* con 2n=2x=16) y la tetraploide *P. spinosa* (2n=4x=32) (Crane y Lawrence, 1952). Esta hipótesis puede explicar la estrecha relación genética encontrada entre los dos grupos de ciruelos.

El polimorfismo detectado en cada grupo de los patrones de *Prunus* es más alto que el polimorfismo observado en melocotonero (Testolin et al., 2000; Aranzana et al., 2002

y Dirlewanger et al., 2002). Estos resultados no son sorprendentes, ya que las especies *P. cerasifera*, *P. insititia* y *P. domestica* son auto-incompatibles (Bernhard et al., 1951) lo que aumenta la heterocigosidad en la especie y, por consiguiente, su variabilidad genética. Además, los híbridos interespecíficos provienen de polinizaciones cruzadas, y son evidentemente más diversos genéticamente que los cultivares de melocotonero, con naturaleza auto-polinizadora.

7.3. ESTUDIO FILOGENÉTICO EN ESPECIES DE *Prunus* MEDIANTE LA TÉCNICA PCR-RFLP

En el Capítulo 6, se ha estudiado el DNA cloroplástico de 62 híbridos ínter-específicos y 22 especies de *Prunus* mediante la técnica de PCR-RFLP, con el fin de detectar relaciones genéticas y filogenéticas entre especies y confirmar la identidad de los parentales maternos de los híbridos ínter-específicos. La técnica de PCR-RFLP es una de las técnicas más fáciles de manejar y permite la evaluación de grandes porciones del genoma cloroplástico de numerosos individuos, en poco tiempo y a bajo coste. Este método ha sido utilizado también en la interpretación de las relaciones filogenéticas en *Prunus* (Badenes y Parfitt, 1995; Panda et al., 2003), y para determinar la filogeografía y la diversidad en poblaciones de *P. spinosa* y *P. avium* (Mohanty et al., 2000; 2001; 2002). En el presente estudio, el análisis del genoma cloroplástico se ha realizado a partir de amplificaciones con tres cebadores universales (DT, HK, K1K2), seguidas de digestiones de los productos de amplificación con tres enzimas de restricción (*Alu*I, *Hinf*I, y *Taq*I), lo que dió lugar a un polimorfismo del tamaño de los fragmentos de restricción. Este tipo de polimorfismo es el resultado de una serie de mutaciones que ocurren a nivel de los lugares de restricción. Las mutaciones son, en general, de tipo inserción o deleción de unas pocas bases. La combinación de todas las mutaciones a nivel de los sitios de restricción, dió lugar a un total de 33 haplotipos para las 84 accesiones estudiadas.

De los 33 haplotipos encontrados, 27 se mostraron específicos para una única especie, lo que demuestra que la técnica puede ser muy interesante para la caracterización molecular en especies *Prunus*. Por otra parte, se pudo confirmar la identidad de los parentales maternos de una parte de los híbridos inter-específicos, gracias a la conservación y la herencia materna del DNA cloroplástico. En el caso de la

especie *P. salicina*, su haplotipo se encontró en seis híbridos inter-específicos complejos, teniendo *P. salicina* como progenitor materno.

Algunos híbridos inter-específicos mostraron haplotipos diferentes de los supuestos maternos. Estas diferencias sugieren la presencia de variabilidad intra-específica en el DNA cloroplástico. El ejemplo más relevante fue el caso de la especie *P. cerasifera* que mostró 8 haplotipos diferentes, lo que sugiere una gran variabilidad genética dentro de esta especie. Trabajos anteriores ya mencionaron la gran diversidad de *P. cerasifera* (Zohary, 1992). Este tipo de variabilidad se mencionó también para otras especies *Prunus*, como en el caso de *P. spinosa* y *P. avium* (Mohanty et al., 2000; 2001; 2002).

Varios parejas de especies *Prunus* compartieron el mismo haplotipo cloroplástico. Teniendo en cuenta la herencia materna del DNA cloroplástico, este resultado informa sobre la existencia de relaciones maternas entre especies *Prunus* con haplotipos comunes. En este estudio, las especies *P. pumila* y *P. besseyi* mostraron un haplotipo en común que podría explicarse por la estrecha relación filogenética que las une. En efecto, Rehder (1940) define estas dos especies como miembros del subgénero *Cerasus*, sección *Microcerasus*. Una estrecha relación fue también mencionada por Mowrey y Werner (1990) entre dichas especies utilizando los marcadores isoenzimas. En este trabajo se mostró igualmente la existencia de una estrecha relación materna entre *P. dasycarpa* y *P. cerasifera*; *P. fruticosa* y *P. cerasus*; y *P. tenella* y *P. nana*.

El polimorfismo detectado a nivel del DNA cloroplástico permitió la reconstrucción de las relaciones filogenéticas entre las especies *Prunus* estudiadas, mediante los análisis del UPGMA y de parsimonia. Algunas de las relaciones filogenéticas encontradas entre especies, responden bien a filogenias ya establecidas previamente por los taxonomistas. Sin embargo, varias discrepancias también fueron detectadas. El ejemplo más relevante fue el caso de los haplotipos que representan especies del subgénero *Cerasus* sección *Microcerasus,* que se encuentran en el análisis del UPGMA agrupados en el mismo cluster que los haplotipos correspondientes a especies del subgénero *Amygdalus* y *Prunus*. Por el contrario, los demás haplotipos que representan especies del subgénero *Cerasus*, secciones *Eucerasus*, *Mahaleb* y *Pseudocerasus*, están situados en otro grupo distinto. De este modo, la clasificación taxonómica de la sección *Microcerasus*, dentro del subgénero *Cerasus*, establecida por Rehder (1940), no concuerda con el análisis genético. Este tipo de discrepancias han sido mencionadas también por Bortiri et al. (2001) y Lee y Wen (2001), que apuntaron que la sección *Microcerasus* no muestra ninguna relación con el resto del subgénero *Cerasus*. Por otra

parte, Katayama y Uematsu (2005) encontraron que los subgeneros *Amygdalus*, *Prunus* y *Cerasus*, sección *Microcerasus*, forman un grupo compacto con el análisis del UPGMA derivado de los marcadores RFLP y el DNA cloroplástico. Estos resultados concuerdan con los obtenidos en este trabajo.

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CAPÍTULO 8

Conclusiones

1. Se estudiaron 20 SSRs en un conjunto de 30 cultivares de melocotonero, procedentes en su mayoría de la misma región en el Valle Medio del Ebro. Los marcadores SSR identificaron 16 genotipos. La baja heterocigosidad observada y esperada reflejan una estrecha base genética dentro del conjunto de los 30 cultivares estudiados. Esta baja variabilidad genética fue debida a que 14 de los 19 supuestos clones de la misma variedad 'Miraflores' resultaron idénticos para todos los loci. Por otra parte, tres variedades ('Tardío del Pilar', 'Amarillo de Septiembre' y 'Tipo Campiel') se encuentran a una distancia genética pequeña de los 'Miraflores'.

2. Se analizaron 15 SSRs en un conjunto de 94 cultivares de melocotonero, de ellos 64 eran representativos de variedades tradicionales del germoplasma local español y 30 de variedades comerciales procedentes de programas de mejora. De los 15 SSRs utilizados, 8 permitieron la identificación inequívoca de los 94 cultivares estudiados, mostrando un genotipo único para cada cultivar.

3. De los 94 cultivares de melocotonero estudiados, se observó una mayor diversidad genética dentro del grupo del melocotón de carne dura, con respecto a las nectarinas y a las variedades de melocotón de carne blanda, y principalmente entre los cultivares de carne dura españoles y los extranjeros. Los cultivares de melocotón españoles de carne amarilla dura, constituyen una fuente interesante de diversidad genética para su utilización en programas de mejora.

4. La transferibilidad de los marcadores SSR entre especies cercanas permitió, en este caso, el uso de los marcadores desarrollados en melocotonero para la identificación y el análisis de la diversidad genética en patrones de especies frutales de hueso (*Prunus* sp.). Se analizaron 20 SSRs y mostraron una gran variabilidad en los 44 patrones de *Prunus,* clasificados previamente en tres grupos: híbridos de melocotonero x melocotonero y almendro x melocotonero, ciruelos de crecimiento rápido y ciruelos de crecimiento lento. Se observó una mayor diversidad genética entre grupos que dentro de cada grupo de patrones, debido a la variabilidad de los mismos, ya que pertenecen a diferentes clases botánicas, y a varias especies de *Prunus*. Tres de los SSRs (BPPCT001,

CPPCT022 y UDP98-407) analizados permitieron la identificación de todos los patrones estudiados.

5. El análisis del genoma cloroplástico mediante la técnica de PCR-RFLP, dió lugar a un polimorfismo en el tamaño de los fragmentos de restricción. Se obtuvieron un total de 33 haplotipos en las 84 accesiones de *Prunus* estudiadas en todas las combinaciones cebador-enzima de restricción. Además, la técnica permitió la detección de relaciones genéticas y filogenéticas entre especies. Por otra parte, la herencia materna del DNA cloroplástico permitió confirmar la identidad de algunos parentales maternos en los híbridos ínter-específicos estudiados.

6. En este trabajo se detectó una estrecha relación materna entre *Prunus. dasycarpa* y *P. cerasifera*; *P. fruticosa* y *P. cerasus*; y *P. tenella* y *P. nana*. Por otra parte, la clasificación taxonómica de la sección *Microcerasus*, dentro del subgénero *Cerasus*, establecida por Rehder (1940), no resulta congruente con el análisis genético de este estudio.

7. El estudio genético del DNA cloroplástico en especies *Prunus*, mediante la técnica de PCR-RFLP, ayudará a establecer la correcta filogenia dentro del género, hasta ahora basada en las características morfológicas de cada especie. Se sugiere el establecimiento de una nueva filogenia para el género *Prunus*, basándose tanto en los estudios morfológicos como en los moleculares obtenidos recientemente.

ANEXOS

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Molecular characterization of Miraflores peach variety and relatives using SSRs

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Abstract

Some traditional peach varieties, originated from the region of Aragón (Spain), were analysed by SSRs (simple sequence repeats). The aim of this research was to characterize 19 clones related to Miraflores variety, with unknown pedigrees, to assess their genetic diversity and to elucidate their possible relationships with 10 traditional peach varieties. Twenty SSR primer pairs with high levels of polymorphism, which have been previously developed for peach, were used in this study. A total of 46 alleles were obtained for all the microsatellites studied, ranging from one to six alleles per locus, with a mean value of 2.3 alleles per locus. Fourteen SSRs were polymorphic in the set of varieties studied and permitted to distinguish 16 different genotypes out of the 30 initially studied, although fourteen 'Miraflores' clones showed identical gel profiles. The genetic distance matrix was used to construct neighbor joining cluster and to perform principal coordinate analysis which allowed the arrangement of all the genotypes according to their genetic relationships. The genetic relationships among these traditional peach varieties, and in particular among 'Miraflores' clones are discussed. The obtained results confirm that microsatellite markers are very useful for these purpose.

Keywords: Clones; Genetic diversity; Genetic relationships; Microsatellites; 'Miraflores' peach variety; Traditional peach varieties

1. Introduction

Peach (*Prunus persica* (L.) Batsch) is a member of the *Rosaceae*, and it is a diploid specie with a basic chromosome number of $x = 8$ and $2n = 16$. The *Prunus persica* is thought to be originated in China and spread to the rest of the world by means of seeds (Layne, 1987). It is a specie well adapted to temperate and subtropical regions, between latitudes of 30° and 45° North and South (Westwood, 1978). The EU (European Union) is one of the main cultivation regions with approximately 4.5 million metric tonnes annual production of peaches and nectarines (28% of the world production). The four countries: Italy, Spain, Greece, and France, ensure 92.7% of this production (Faostat, 2005), Italy being the greatest peach producer of them (1,750,000 metric tonnes) followed by Spain (1,130,800 metric tonnes). Peach is one of the most economically and socially important deciduous fruit tree species. Therefore, precise cultivar identification and characterisation is essential for improving and securing peach culture in the world.

The official methods used to characterize and identify varieties in fruit tree species are based on morphological characterization and phenological observations according to UPOV (Unité pour la Protection des Obtentions Végétales) and IPGRI (International Plant Genetic Resources Institute) descriptors. This approach requires time, and the morphological characters can be subject to environmental influences. New molecular methods have been incorporated to characterize the varieties at the DNA level (see for a review: Martínez-Gómez et al., 2003). Molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been used for genetic analysis and the construction of genetic linkage maps (Gogorcena et al., 1993; Warburton and Bliss, 1996; Ortiz et al., 1997; Bartolozzi et al., 1998; De Vicente et al., 1998; Casas et al., 1999). On the downside, these DNA markers either present low reproducibility (RAPDs), or are time consuming and expensive (RFLPs, AFLPs). In the last years, microsatellite markers (SSR) are becoming the appropriate marker for molecular characterization and genetic diversity studies (Fang and Roose, 1997;

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Alvarez et al., 2001; Huang et al., 2002; Rallo et al., 2003; Moussaoui, 2005). Consisting of tandem repeats of mono-, bi-, tri- or tetra-nucleotides in the eukaryotic genome, these molecular markers present a very high polymorphism based on the number of the nucleotide motif repeats (Morgante and Olivieri, 1993). Besides polymorphism, they have a codominant inheritance, they are reproducible and easily detected by PCR. Microsatellite marker primer pairs have been developed in peach (Sosinski et al., 2000; Testolin et al., 2000; Aranzana et al., 2002; Dirlewanger et al., 2002), and then used in genetic diversity analyses for sweet cherry (Dirlewanger et al., 2002), peach (Aranzana et al., 2003), and apricot (Romero et al., 2003; Zhebentyayeva et al., 2003).

'Miraflores' is a Spanish native peach variety, with attractive characteristics, especially high yields and good quality of fruits (Moreno, 2005). This nonmelting and clingstone variety is widely used for the fresh market as well as for processing. This variety appeared in 1970s at the Jalón valley (Aragón), one of the most important deciduous fruit tree growing areas in Spain. 'Miraflores' has unknown parents, although it is believed that it could originate from 'Campiel' seedlings, a traditional variety locally cultivated in this area (De Asso, 1798). The correct molecular characterization of this variety should prevent all confusion with other varieties with similar morphological characteristics, and enhance its use in peach breeding programs. On the other hand, this will be useful to warrant this variety as a specific typical product with a designation of origin.

Therefore, the aim of this study was to characterize 19 clones of 'Miraflores' peach variety with unknown pedigree, using 20 SSR primer pairs. Ten old Spanish peach varieties originated from the same region were also included in this investigation, to check genetic similarities and possible genetic relationships with 'Miraflores'.

2. Materials and methods

2.1. Plant material

Thirty peach accessions, being either clones of 'Miraflores' or closely related varieties with other traditional peach varieties were collected throughout Aragón, and were analysed in this study. Nineteen accessions (clones and/or closely related varieties) were provided from a recent survey of 'Miraflores' variety ('Miraflores' 1–19). The other eleven accessions are old Spanish peach varieties originated from the same region of Aragón (Spain); two clones of 'Tipo Campiel' variety ('Tipo Campiel 1' and 'Tipo Campiel 2') and nine traditional cultivars ('Amarillo de Calanda', 'Zaragozano Amarillo', 'Amarillo de Gallur', 'Tardío del Pilar', 'Oropel', 'Zaragozano Rojo', 'Maluenda', 'Bonet IV', 'Amarillo de Septiembre'). From all accessions, young leaves were collected for DNA extraction.

2.2. Genomic DNA extraction

Fresh young leaves were ground to powder with liquid N_2 using a mortar and pestle. Genomic DNA was isolated from leaf samples using a CTAB (cethyltrimethyl ammonium bromide) extraction method (Cheng et al., 1997). The extraction buffer contained 2% CTAB, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris pH 8.0, and 0.4% 2-mercaptoethanol. Samples were incubated at 65 °C for approximately 30–60 min, mixed with an equal volume of chloroform–isoamyl alcohol (24:1), and centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was transferred to a clean microcentrifuge tube and treated with RNAse A (10 mg/ml, 60 min, 37 °C), then mixed with an equal volume of cold isopropanol. The DNA was pelleted by centrifugation at 13,000 rpm for 3–5 min. The supernatant was removed and the DNA pellet washed with 500 μl of 70% ethanol and 50 μl of wash buffer (3 M sodium acetate, 0.1 M magnesium acetate pH 8.0), to remove residual CTAB, salt, and other contaminants. The pellet was dried for 1 h and then dissolved in 100 μl of TE buffer (10 mM Tris–HCl, 100 mM EDTA, pH 8.0). DNA quality was examined by electrophoresis in 0.8% agarose and DNA concentration was quantified spectrophotometrically (Gene Quant, Amersham Pharmacia Biotech). The extracted DNA was diluted to 5 ng/μl with TE buffer and stored at −20 °C for PCR amplifications.

2.3. DNA amplification

Twenty SSR markers were studied (Table 1) using primer pairs previously developed for peach. Amplification reactions were carried out in a final volume of 15 μl containing 10 ng of template DNA, 1× reaction buffer (20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl pH 8.8), 2 mM $MgCl_2$, 0.2 mM of dNTPs (50 μM of each) (Amersham Pharmacia Biotech), 0.15 μM of forward and reverse primers each, and 0.5 U of Tth DNA Polymerase (Biotools Band M Labs, S.A., Spain), overlaid with a drop of mineral oil (Sigma, St. Louis, MO). The PCR amplifications were carried out on a Gene Amp 2700 thermocycler (Applied Biosystems) using the following temperature cycles: 1 cycle of 3 min at 95 °C; 35 cycles of 1 min at 94 °C, 45 s at the corresponding annealing temperature (Table 1) and 1 min at 72 °C. The last cycle was followed by a final incubation for 7 min at 72 °C and the PCR products were stored at 4 °C before analysis. Two independent SSR reactions were performed for each DNA sample. The DNA amplification products were loaded on 5% polyacrilamide sequencing gels. Gels were run for 2 h at 65 W. The gels were silver-stained according to the protocol described by Bassam et al. (1983). Fragment sizes were estimated with the 30–330 bp AFLP ladder (Invitrogen, Carlsbad, CA) DNA sizing markers, and analysed by the Quantity One program (Bio Rad, Hercules, CA).

2.4. Data analysis

The number of alleles per locus was counted from the gel profile analysis. The observed heterozygosity (H_o) was calculated for each locus as the number of heterozygous individuals over the total number of individuals analysed. The expected heterozygosity was calculated as $H_e = 1 - \sum p_i^2$, where p_i is the frequency of ith allele (Nei, 1973). The power of discrimination was calculated as $PD = 1 - \sum g_i^2$, where g_i is the frequency of ith genotype (Kloosterman et al., 1993). Those

Table 1
List of the 20 SSR primers used in this study, size range, annealing temperature, number of alleles and variability parameters

Locus code	References	Size range (bp)	T_a (°C)	N	H_o	H_e	#Genotypes	PD
CPPCT002	Aranzana et al. (2002)	102	58	1	0	0	1	0
CPPCT004	Aranzana et al. (2002)	262–277	56	2	0.03	0.10	2	0.13
CPPCT005	Aranzana et al. (2002)	154	58	1	0	0	1	0
CPPCT006	Aranzana et al. (2002)	182–192	60	2	0.70	0.52	3	0.46
CPPCT017	Aranzana et al. (2002)	183	60	1	0	0	1	0
CPPCT022	Aranzana et al. (2002)	248–292	58	3	0	0.13	3	0.13
CPPCT028	Aranzana et al. (2002)	134–138	58	3	0	0.35	3	0.35
CPPCT029	Aranzana et al. (2002)	192–196	58	2	0	0.43	2	0.43
CPPCT030	Aranzana et al. (2002)	186	56	1	0	0	1	0
CPPCT033	Aranzana et al. (2002)	151–153	58	2	0.10	0.09	2	0.18
UDP98022	Testolin et al. (2000)	124–138	64	3	0	0.52	3	0.52
UDP98025	Testolin et al. (2000)	134–142	65	2	0.13	0.44	3	0.54
UDP98407	Testolin et al. (2000)	174–198	60	2	0.57	0.40	3	0.59
UDP98408	Testolin et al. (2000)	102	56	1	0	0	1	0
BPPCT001	Dirlewanger et al. (2002)	152–166	60	6	0.03	0.62	7	0.63
BPPCT007	Dirlewanger et al., 2002	146	58	1	0	0	1	0
BPPCT008	Dirlewanger et al. (2002)	100–156	59	4	0.67	0.67	8	0.67
BPPCT015	Dirlewanger et al. (2002)	168–222	62	5	0.03	0.50	6	0.52
BPPCT017	Dirlewanger et al. (2002)	162–174	60	2	0.57	0.54	2	0.57
BPPCT038	Dirlewanger et al. (2002)	127–129	62	2	0.67	0.45	2	0.45
Mean				2.3	0.18	0.29	2.75	0.31

Note: N, number of alleles; T_a, annealing temperature; H_o, observed heterozygosity; H_e, expected heterozygosity; #Genotypes; different genotypes per locus; PD, power of discrimination.

parameters served to evaluate the information given by the microsatellite markers (Table 1).

Data were analyzed using the NTSYS-pc, version 2.1 program (Rohlf, 2000). A 0/0.5/1 (absence/allele in heterozygosity/allele in homozygosity) matrix was constructed. The genetic distances between pairs of varieties were estimated from the matrix with the SIMGEND module using the Nei coefficient (Nei, 1972). Cluster analyses were carried out using neighbor joining (NJ) algorithm (Saitou and Nei, 1987). The resulting cluster was represented as a dendrogram. A principal coordinate analysis based on the similarity matrix was also performed.

3. Results

3.1. Microsatellites diversity

Spanish peach varieties were analysed with 20 SSRs. All the SSRs studied were single-locus and produced alleles that could be scored, with a total of 46 ranging from one to six per locus, with a mean value of 2.3 alleles per locus. Fourteen out of the 20 SSRs employed, were polymorphic and it was possible to distinguish unambiguously 16 peach genotypes from the 30 accessions studied. However, six SSR loci (CPPCT002, CPPCT005, CPPCT017, CPPCT30, UDP98408 and BPPCT007) were monomorphic for the peach plant material evaluated (Table 1).

Observed heterozygosity (H_o) ranged from 0 to 0.70, with a mean value of 0.18. The expected heterozygosity (H_e) ranged from 0, for the monomorphic SSRs, to 0.67 in CPPCT006, with a mean value of 0.29. The power of discrimination (PD) varied from 0, for the monomorphic loci, to 0.67 in BPPCT008, with an average value of 0.31. Differences were found in the number of genotypes identified per locus. This number varied between one unique genotype in the monomorphic loci, to eight genotypes using BPPCT008 with a mean value of 2.75 (Table 1).

The five most polymorphic microsatellites with the highest H_e and PD were BPPCT001, BPPCT015, BPPCT008, UDP98022, and CPPCT028 (Table 1). They allowed the unambiguous discrimination of the same 16 accessions separated using the 20 SSRs. These five SSR primer pairs detected a total of 21 alleles, ranging from 3 to 6 alleles per locus and with a mean value of 4.2 alleles per locus. The expected heterozygosity mean value for these microsatellites was $H_e = 0.53$, and the power of discrimination mean value was PD = 0.54.

3.2. Cluster and principal coordinate analysis

The genetic distance (D) among the different genotypes studied using the 20 SSRs was reproduced in the neighbor joining (NJ) dendrogram (Fig. 1), according to the original data obtained in the similarity matrix, and based on the additive genetic distances among the genotypes (Saitou and Nei, 1987).

The dendrogram generated from the NJ cluster analysis showed two main groups. The first group was composed of the two 'Tipo Campiel' clones, 'Tardío del Pilar', 'Amarillo de Septiembre' and all the 'Miraflores' clones but 'Miraflores 3', which was clustered out of the two main groups. The second group included the rest of the studied varieties. In the first group, fourteen 'Miraflores' clones ('Miraflores' 5 to 11 and 'Miraflores' 13 to 19) were clustered together with a null genetic distance among them. The 'Miraflores 1' and 'Miraflores 2' were closely related to the 14 indistinguishable

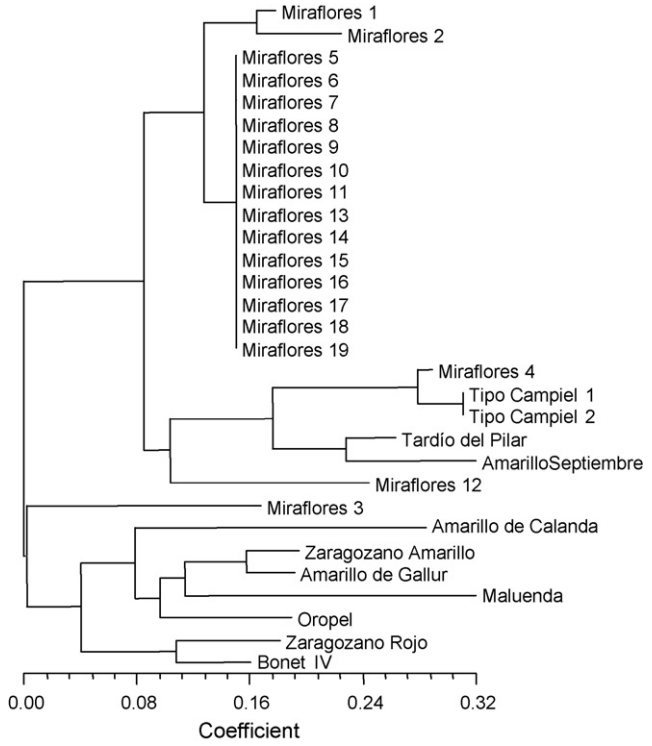

Fig. 1. Dendrogram of the 30 peach accessions obtained from the neighbor joining analysis (NJ) using Nei's genetic distance (1972) after amplification with 20 SSR primer pairs.

'Miraflores' clones with a genetic distance $D = 0.07$ and 0.12, respectively. However, 'Miraflores 4' and 'Miraflores 12' were clustered further away, at a genetic distance of $D = 0.26$ and 0.21, respectively. The two 'Tipo Campiel' clones were genetically identical with the 20 SSRs, and 'Miraflores 4' was closely related to them with a small genetic distance of $D = 0.04$.

The principal coordinate analysis (PCA) generated two clearly significant components, which explained 44 and 32% of the total variance (Fig. 2). This analysis showed well-defined distribution patterns of the accessions, according to the genetic distances and the relationships among them. In the PCA, two main groups could be clearly seen, the first one was composed by 'Amarillo de Septiembre', 'Tardío del Pilar' and all the 'Miraflores' clones but 'Miraflores 3' and 'Miraflores 4'. The second group included the varieties 'Oropel', 'Amarillo Gallur', 'Zaragozano Amarillo' and 'Amarillo de Calanda'. Again, the PCA showed the small distance between the 'Miraflores 4' and the two 'Tipo Campiel' clones. The 'Miraflores 3' appeared further away from all the 'Miraflores' clones.

4. Discussion

4.1. Genetic diversity

The 20 SSR loci produced between 1 and 6 alleles with a mean value of 2.3 alleles per locus. This mean value calculated with 30 peach accessions, was lower than the mean values

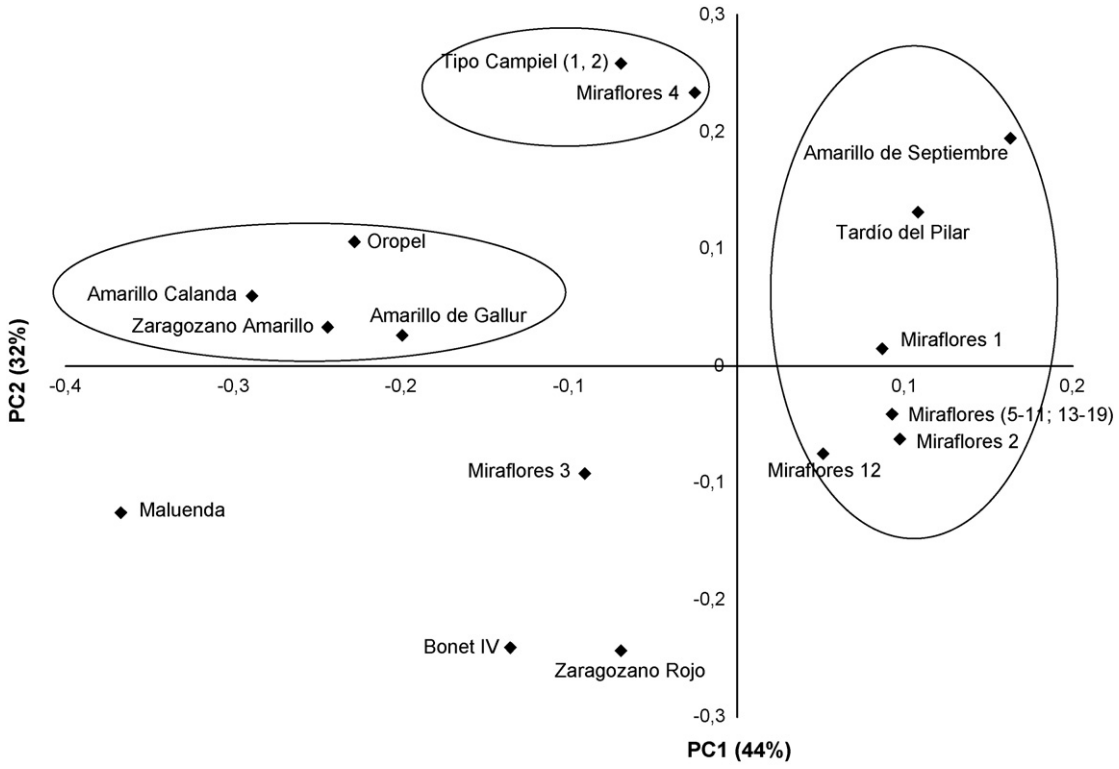

Fig. 2. Plot of the first two components (PC1 and PC2) of the principal coordinate analysis on the similarity matrix for 30 peach accessions (varieties and related clones) using 20 SSRs.

obtained by other authors in this species. Testolin et al. (2000) mentioned a value of 4.5 for a set of 50 varieties analysed with 26 microsatellites. Aranzana et al. (2002) cited 3.2 for a set of 25 varieties with 24 SSRs, and Dirlewanger et al. (2002) observed 4.2 for a set of 27 varieties with 41 SSR primer pairs. Mean values for the expected heterozygosity (H_e) and for the power of discrimination (PD) were also lower than the values recorded by these authors. In the present study, the H_e and PD were 0.29 and 0.31, respectively. Testolin et al. (2000) mentioned $H_e = 0.47$ and PD = 0.60; Aranzana et al. (2002) found value of 0.41 for H_e, and 0.60 for PD, and Dirlewanger et al. (2002) reported values of 0.41 for H_e, and 0.54 for PD. These results indicate a low variability in our plant material that can be explained by the close genetic relationship among the peach accessions studied and in particular, among the 'Miraflores' clones. However, using only the five most polymorphic primer pairs (BPPCT001, BPPCT008, BPPCT015, UDP98022, and CPPCT028), the mean value of alleles per locus increased to 4.2. An increase in the mean values of the expected heterozygosity ($H_e = 0.52$) and the power of discrimination (PD = 0.53) has also been shown. These new values are closer to the ones mentioned by Testolin et al. (2000), Aranzana et al. (2002) and Dirlewanger et al. (2002). The high levels of polymorphism in the selected five SSRs, permitted to distinguish unambiguously 16 genotypes within the thirty studied accessions, the same genotypes that were discriminated using all the twenty SSR primers pairs. Thus, those five polymorphic SSRs are interesting markers for studies aimed at distinguishing highly related peach varieties. The fourteen clones of 'Miraflores' showed identical patterns for all the SSRs used in this study. These clones either belong to the same clone or represent different clones that differ by a single or few gene mutations, which could not be detected by SSR analysis (Testolin et al., 2000).

4.2. Cultivar relationships from cluster and principal coordinate analysis

The fourteen 'Miraflores' clones showed identical SSR profiles and were clustered together in the NJ tree. These clones have also similar pomological characteristics (M.A. Moreno, unpublished observation). The genetic similarity among some 'Miraflores' clones was also shown in a preliminary study reported by Moreno and Casas (2002) using 10 SSRs and 7 RAPDs. In terms of genetic distance, the 'Miraflores 1' and 'Miraflores 2' were closely related with the group of the fourteen identical 'Miraflores' clones with a genetic distance of 0.07 and 0.12, respectively. These two clones differed from the 'Miraflores' main group in only two SSR loci. The 'Miraflores 1' had a new allele with +2 bp in the locus UDP98022 (138 bp) and the lack of the allele 174 bp in the locus BPPCT017. The 'Miraflores 2' had a new allele with +2 bp in the locus UDP98022 (138 bp) and a new allele with +2 bp in the locus CPPCT028 (136 bp). These two clones presented also similar *Rosaceae* flower shape, the same flowering and ripening date, and similar yield and fruit characteristics with the group of identical 'Miraflores' clones. The small molecular discrepancies of these two clones from the main group of 'Miraflores' may have been caused by SSR mutations as it was also suggested by Aranzana et al. (2003).

The 'Miraflores 12' is closer to the 'Miraflores' group ('Miraflores' 5–11 and 13–19) than to the old Spanish varieties at a genetic distance of $D = 0.21$. Moreover, this clone shared at least one allele in 17 SSR loci (34 alleles out of the 40 studied) with the group of identical 'Miraflores' clones. These results point out the existence of a close parental relationships between these clones and we can suggest that 'Miraflores 12' could be one of the 'Miraflores' progenitors. 'Miraflores 4' was clustered to the identical 'Miraflores' group at a genetic distance of $D = 0.26$. This clone is closely related to the two identical 'Tipo Campiel' clones at a genetic distance of $D = 0.04$. 'Miraflores 4' had at least one common allele in all SSR loci with the 'Tipo Campiel' clones and shared 37 alleles of the total alleles studied. According to the morphological data (data not shown), 'Miraflores 4' presents similar fruit characteristics to 'Tipo Campiel' cultivar (high percentage of redness in the skin), and similar flower morphology (bell flower shape). Moreover, the pulp close to the stone of the fruit is red as in the 'Tipo Campiel' fruits. Our findings confirm the hypothesis reported previously by Moreno and Casas (2002) that 'Miraflores 4' could be a seedling of 'Tipo Campiel' cultivar.

Finally, 'Miraflores 3' was clustered out of the two main groups shown in the NJ tree, at a genetic distance of $D = 0.4$. Furthermore, 'Miraflores 3' showed chemical and physical characteristics different from the rest of 'Miraflores' clones (fruit shape, yellow-red skin appearance, early ripening, sugar and acids content, etc.). This clone probably belongs to another variety which has morphological and phenological characteristics similar to those of 'Miraflores' variety. Previous work pointed out that this clone could be the 'Godina 58GC76' variety, based on similarities in fruit characteristics (fruit color, fruit shape, sugar and acid level etc.) (M.A. Moreno, unpublished observation).

The 'Miraflores' clones studied in this work have unknown parents and pedigrees. Old spanish varieties were included in the study to check an eventual relationship with 'Miraflores' clones. As it is shown in the dendrogram (Fig. 1), and the PCA (Fig. 2), the varieties 'Tardío del Pilar', 'Amarillo de Septiembre', 'Tipo Campiel' and all of the 'Miraflores' clones, but 'Miraflores 3', belong to the same group. The varieties 'Tardío del Pilar', 'Amarillo de Septiembre', and 'Tipo Campiel' were related to the 'Miraflores' clones at the genetic distances of 0.24, 0.30 and 0.31, respectively. In fact, these varieties, showed similar physical fruit characteristics (properties of the pulp and skin appearance) to those found in 'Miraflores' clones. Thus, we hypothesise that they could be close relatives of 'Miraflores' cultivar. However, the rest of the old Spanish varieties ('Amarillo de Calanda', 'Zaragozano Amarillo', 'Amarillo Gallur', 'Oropel', 'Zaragozano Rojo', 'Maluenda' and 'Bonet IV'), were clustered in another main group far away from the 'Miraflores' clones.

In this work, the absence of SSR differences among fourteen clones of 'Miraflores' could be explained by the usual clonal propagation of 'Miraflores' carried out by nurseries and fruit

growers. Another possibility could be that these clones may represent the original genotype of 'Miraflores' with few genetic variations such as sport mutations. However by using SSRs, it is highly improbable to distinguish mutants that differ from the original genotype in one or few genes (Botta et al., 1995; Moreno et al., 1998; Aranzana et al., 2003).

In conclusion, we regard the clones (1, 2, 5–11 and 13–19) of 'Miraflores' as true clones of the 'Miraflores' variety, and they will be included in future programs of selection. While 'Miraflores 12' was considered as one of the 'Miraflores' progenitors and 'Miraflores 4' could be considered as a new cultivar, closely related to 'Tipo Campiel'. 'Miraflores 3' is considered unrelated to the main group of 'Miraflores'. These studies make possible the adequate choice of clones and varieties in new crosses for selection purposes.

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