Genetic Diversity and Structure in a Collection of Ancient Spanish Pear Cultivars Assessed by Microsatellite Markers

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ABSTRACT. A collection of 141 old and local Spanish accessions of pear (*Pyrus communis*) from the Escuela Técnica Superior de Ingeniería Agraria-Universidad de Lleida (ETSIA-UdL) Pear Germplasm Bank in Lleida, Spain, were studied using a set of eight microsatellite markers to estimate the genetic diversity of the collection, to identify the genetic structure and relationships among its accessions, and to establish a representative core collection. An additional set of 13 well-known pear cultivars, currently grown in Spain and which represent a wide genetic diversity, were added as reference. The eight simple sequence repeat (SSR) loci amplified 97 alleles, with nine to 15 alleles per locus, and with the expected heterozygosity ranging from 0.65 to 0.89. All of the accessions except for 16 had at least one of the 48 rare alleles (frequency < 0.05) identified, and seven unique alleles were found in six accessions. Fifteen accessions were identified as synonyms and were excluded from the analysis. Genetic analyses performed by hierarchical clustering, Bayesian model-based clustering, and factorial correspondence analysis supported the existence of three groups among the accessions with moderate [fixation index (F_{ST}) = 0.074], but significant, differentiation. As a whole, most of the germplasm (about 75%) curated at the collection showed its genetic distinctness with respect to the main pear cultivars used in European orchards. In fact, most reference cultivars were included in one single cluster that, moreover, had the lowest genetic diversity and allelic richness, in spite of having been chosen as heterogeneous material from different origins. The obtained results were also used to create a core collection with 35 accessions constituting an efficient and accessible entry point in the ETSIA-UdL pear collection for breeding and research communities.

Pear (*Pyrus* spp.) is one of the most important fruit crops of the Rosaceae family, ranking second to apple (*Malus ×domestica*) in world and European production of pome fruit tree species (Food and Agriculture Organization of the United Nations, 2010). *Pyrus communis* is the most commonly cultivated pear species in Europe, America, and Africa, whereas *Pyrus pyrifolia* is the main cultivated species in Asia (Bell, 1991). Worldwide production of *P. communis* pears is based upon relatively few cultivars, most of them selected in late 18th and 19th century, or derived from those. Furthermore, the genetic base of cultivated pear in western Europe has significantly narrowed in the last few years. In 1986, seven cultivars accounted for ≈58% of pear production in western Europe (Bell, 1991), but nowadays, seven cultivars account for 75% of the production (World Apple and Pear Association, 2009). ‘Conference’ has become predominant in most European producing countries, and accounts for nearly one-third of European Union production. The reasons for this concentration are varied, but include economic and market factors, changes in consumption patterns, and biological aspects, such as productivity, storage ability, and susceptibility to pests and diseases (Bell, 1991).

As a consequence, many of the traditional or local cultivars have been considered obsolete and replaced, leading to a dramatic loss of genetic diversity. The recognition of the need for the collection and preservation of endangered fruit germplasm has encouraged the establishment of genetic resource conservation programs. In 1986, a germplasm bank of old and local pear cultivars was established by the Horticulture section of the ETSIA-UdL. This germplasm bank currently maintains 114 *Malus* and 169 *Pyrus* accessions, collected at 12 northern Spanish provinces (Urbina et al., 2007), at the Estación Experimental de Lleida of the Institut of Recerca i Tecnologia Agroalimentària (IRTA) in Lleida, Spain. In 2002, a research program was launched to evaluate the genetic diversity of the collection through detailed morphological and agronomical description, and fingerprinting analysis based upon molecular markers was initiated in 2005.

Microsatellite or simple sequence repeat (SSR) markers have been favored over other methods in establishing unique genetic identities or fingerprints and in assessing genetic diversity within a collection due to their high polymorphism level, reproducibility, and relative ease of analysis (Schlötterer, 2004). Moreover, SSR have also proven useful in creating core collections that represent not only the genetic structure of germplasm collections, but also their phenotypic structure (Santesteban et al., 2009). The SSR markers used in the earliest studies in pear were derived from apple (Yamamoto et al., 2001; Hemmat et al., 2003), as apple proved to be highly conserved in pear. However, after the development of SSR derived from asian pear (*P. pyrifolia*) and

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Table 1. Pear accessions maintained by the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) Germplasm Bank and reference cultivars that were included in the study, in alphabetical order. Accessions selected to constitute the core collection are indicated in bold. Collection information includes accession name and bank code, site of collection, specific latitude and longitude, approximate elevation, and collecting source code according to Food and Agriculture Organization of the United Nations/International Plant Genetic Resources Institute (FAO/IPGRI, 2001) multicrop passport descriptors.

Accession name ^z	Accession code	Site of collection in Spain	Latitude	Longitude	Elevation (m)	Collecting source ^y
<i>Agramunt - 1</i>	PRF-135	Puigvert d'Agramunt, Lleida	41.78	1.13	360	23
<i>Agua</i>	PRF-030	Nalda, La Rioja	42.33	-2.48	530	23
<i>Agua Iglesia</i>	PRF-028	Lardero, La Rioja	42.42	-2.45	435	23
<i>Agua Magistral</i>	PRF-029	Lardero, La Rioja	42.40	-2.47	460	23
<i>Aguilar - 1</i>	PRF-011	Aguilar de Campoo, Palencia	42.78	-4.25	900	23
<i>Almenar - 1</i>	PRF-071	Almenar, Lleida	41.77	0.62	270	62
<i>Angelina</i>	PRF-141	Isona i Conca Dellà, Lleida	42.08	1.12	1000	23
<i>Areny - 1</i>	PRF-091	Areny de Noguera, Huesca	42.25	0.73	670	62
<i>Areny - 2</i>	PRF-092	Areny de Noguera, Huesca	42.25	0.73	675	62
<i>Areny - 3</i>	PRF-093	Areny de Noguera, Huesca	42.25	0.73	675	62
<i>Areny - 4</i>	PRF-113	Areny de Noguera, Huesca	42.25	0.72	750	23
<i>Azucar verde - 1</i>	PRF-007	Albelda de Iregua, La Rioja	42.37	-2.47	490	21
<i>Azucar verde - 2</i>	PRF-008	Albelda de Iregua, La Rioja	42.37	-2.47	490	21
<i>Balaguer - 1</i>	PRF-066	Balaguer, Lleida	41.75	0.77	220	23
<i>Balaguer - 2</i>	PRF-067	Balaguer, Lleida	41.75	0.77	220	23
<i>Balaguer Flix - 1</i>	PRF-046	Balaguer, Lleida	41.73	0.93	290	62
<i>Balaguer Flix - 2</i>	PRF-047	Balaguer, Lleida	41.73	0.93	290	23
<i>Balaguer Flix - 3</i>	PRF-048	Balaguer, Lleida	41.73	0.93	290	23
<i>Balaguer Flix - 4</i>	PRF-049	Balaguer, Lleida	41.73	0.93	290	23
<i>Balaguer Flix - 5</i>	PRF-050	Balaguer, Lleida	41.73	0.93	290	23
<i>Balaguer Flix - 6</i>	PRF-051	Balaguer, Lleida	41.73	0.93	290	23
<i>Balart</i>	PRF-070	Almenar, Lleida	41.77	0.58	280	23
<i>Benavent de la Conca - 1</i>	PRF-142	Benavent de la Conca, Lleida	42.08	1.12	985	23
<i>Bergamota</i>	PRF-112	Sopeira, Huesca	42.33	0.73	780	23
<i>Bescanó - 1</i>	PRF-121	Bescanó, Girona	41.97	2.75	110	23
<i>Bescanó - 2</i>	PRF-122	Bescanó, Girona	41.97	2.72	110	23
<i>Biscarri - 1</i>	PRF-143	Biscarri, Lleida	42.12	1.12	920	23
<i>Blanca d'aigua</i>	PRF-079	Baix Pallars, Lleida	42.33	1.00	1075	25
<i>Blanquilla durró</i>	PRF-102	La Vall de Boí, Lleida	42.48	0.82	1480	23
<i>Blanquilla rosada</i>	PRF-058	Huesa del común, Teruel	41.05	-0.93	820	21
<i>Bodegas Lardero</i>	PRF-017	Lardero, La Rioja	42.42	-2.47	435	23
<i>Bossost - 1</i>	PRF-107	Bossòst, Lleida	42.78	0.68	720	23
<i>Bossost - 2</i>	PRF-108	Bossòst, Lleida	42.78	0.68	720	23
<i>Campanilla Lardero</i>	PRF-018	Lardero, La Rioja	42.42	-2.47	435	23
<i>Castelldans - 1</i>	PRF-130	Castelldans, Lleida	41.52	0.77	240	62
<i>Clota</i>	PRF-042	Miralcamp, Lleida	41.60	0.90	260	62
<i>Coll - 1</i>	PRF-036	Barruera, Lleida	42.47	0.78	1180	23
<i>Coll - 2</i>	PRF-037	Barruera, Lleida	42.47	0.78	1180	23
<i>Coll - 3</i>	PRF-038	Barruera, Lleida	42.47	0.78	1180	23
<i>Colorada de juliol</i>	PRF-081	Lleida, Lleida	41.62	0.60	185	23
<i>Cor podrit</i>	PRF-072	Almenar, Lleida	41.77	0.62	270	62
<i>Coure</i>	PRF-116	Olot, Girona	42.15	2.55	560	40
<i>Cristóbal de Bejar - 1</i>	PRF-039	Cristóbal, Salamanca	40.45	-5.87	870	23
<i>De Agua de agosto</i>	PRF-061	Valls de Aguilar, Lleida	42.22	1.35	530	62
<i>De Batre</i>	PRF-059	Els Alamus, Lleida	41.62	0.65	230	62
<i>De Campana</i>	PRF-083	Castell de Mur, Lleida	42.10	0.90	370	23
<i>De Cuixa</i>	PRF-062	Valls de Aguilar, Lleida	42.22	1.35	540	62
<i>De invierno</i>	PRF-111	Vielha e Mijaran, Lleida	42.70	0.78	1040	23
<i>Donguindo</i>	PRF-012	Fuenmayor, La Rioja	42.45	-2.55	435	23
<i>Donguindo</i>	PRF-009	Lardero, La Rioja	42.42	-2.45	435	21
<i>Durro - 1</i>	PRF-101	Durró, Lleida	42.48	0.82	1460	23
<i>Durro - 2</i>	PRF-103	Durró, Lleida	42.48	0.82	1470	23
<i>Durro - 3</i>	PRF-104	Durró, Lleida	42.48	0.82	1470	23
<i>Durro - 4</i>	PRF-105	Durró, Lleida	42.48	0.82	1425	23

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Table 1. Continued.

Accession name ^z	Accession code	Site of collection in Spain	Latitude	Longitude	Elevation (m)	Collecting source ^y
Erill la Vall - 1	PRF-139	Durró, Lleida	42.52	0.83	1245	28
<i>Espot - 1</i>	PRF-075	Espot, Lleida	42.58	1.10	1315	23
Extragüña	PRF-021	Panes, Asturias	43.32	-4.58	40	23
Flor de invierno	PRF-064	Alpicat, Lleida	41.65	0.57	270	21
Fresquilla Lardero	PRF-006	Lardero, La Rioja	42.42	-2.47	440	23
Fulla de roure	PRF-060	Noves de Segre, Lleida	42.22	1.35	530	62
Gitaneta	PRF-044	Juneda, Lleida	41.53	0.82	240	23
<i>Gotarta - 1</i>	PRF-097	Pont de Suert, Lleida	42.42	0.75	1180	23
<i>Gotarta - 2</i>	PRF-098	Pont de Suert, Lleida	42.42	0.75	1160	23
<i>Isla - 1</i>	PRF-005	Isla, Santander	43.48	-3.57	15	23
La Guardia - 1	PRF-084	Castell de Mur, Lleida	42.10	0.87	395	61
<i>Les - 1</i>	PRF-106	Les, Lleida	42.82	0.72	615	23
Limón (de Limón)	PRF-054	Huesa del común, Teruel	41.02	-0.92	800	23
Limonera Francesa	PRF-019	Lleida, Lleida	41.62	0.63	145	21
Limonera Mutalbarrec	PRF-020	Albatàrrec, Lleida	41.57	0.60	150	21
Limonera Mutbarbens	PRF-137	Barbens, Lleida	41.67	0.98	270	21
Llei de Mau	PRF-123	Brunyola, Girona	41.92	2.65	230	23
Lombardia (Cuchillo)	PRF-015	Lardero, La Rioja	42.43	-2.45	410	23
Mala cara	PRF-055	Huesa del común, Teruel	41.02	-0.92	800	23
Mantecosa de Valencia	PRF-003	Lardero, La Rioja	42.42	-2.45	435	23
Montcortes - 1	PRF-089	Montcortes, Lleida	42.33	1.00	1080	23
<i>Montcortes - 2</i>	PRF-090	Montcortes, Lleida	42.32	1.00	1060	61
<i>Mont-ros - 1</i>	PRF-087	Torre de Capdella, Lleida	42.42	0.98	900	23
<i>Navalmoral de Bejar - 1</i>	PRF-040	Navalmoral de Bejar, Salamanca	40.40	-5.77	810	23
<i>Navalmoral de Bejar - 2</i>	PRF-140	Navalmoral de Bejar, Salamanca	40.40	-5.77	810	23
<i>Noales - 1</i>	PRF-132	Noales, Huesca	42.47	0.70	1045	23
<i>Noales - 2</i>	PRF-133	Noales, Huesca	42.47	0.70	1050	23
<i>Noales - 3</i>	PRF-134	Noales, Huesca	42.45	0.68	1240	23
<i>Organya - 1</i>	PRF-063	Organya, Lleida	42.22	1.35	575	23
Ortiguero	PRF-024	Panes, Asturias	43.48	-4.58	40	23
Pela de Roure	PRF-065	Balaguer, Lleida	41.75	0.77	220	23
Pell de Galàpet	PRF-117	Olot, Girona	42.15	2.55	560	40
Peñon	PRF-023	Colunga, Asturias	43.47	-5.27	35	23
Pera d'aigua	PRF-120	Sant Julià de Ramis, Girona	42.03	2.87	50	23
Pera de Cardós	PRF-088	La Torre de Cabdella, Lleida	42.33	0.97	820	23
Pera d'estiu	PRF-124	Sant Esteve Sesrovires, Barcelona	41.50	1.87	190	23
Pera d'hivern	PRF-078	Baix Pallars, Lleida	42.33	1.00	1075	23
Pera d'hivern	PRF-085	Castell de Mur, Lleida	42.12	0.87	390	62
Pera Lloquera	PRF-114	Bellver de Cerdanya, Lleida	42.40	1.77	1480	23
Perot	PRF-099	Pont de Suert, Lleida	42.42	0.75	1160	23
<i>Piedrahita - 1</i>	PRF-004	Piedrahita, Avila	40.45	-5.32	1065	23
<i>Piedrahita - 2</i>	PRF-076	Piedrahita, Ávila	40.45	-5.32	1065	23
<i>Puigcerdà - 1</i>	PRF-115	Puigcerdà, Girona	42.42	1.90	1140	12
Reguard - 1	PRF-086	Reguard, Lleida	42.28	0.92	690	23
Rengá	PRF-082	Castell de Mur, Lleida	42.10	0.88	380	23
Roma	PRF-010	Lardero, La Rioja	42.42	-2.45	430	21
Roma	PRF-045	Puig Gròs, Lleida	41.55	0.88	280	21
<i>Rosal - 1</i>	PRF-032	O Rosal, Pontevedra	41.92	-8.83	45	23
<i>Rosal - 2</i>	PRF-033	O Rosal, Pontevedra	41.92	-8.83	45	23
<i>Rosal - 3</i>	PRF-034	O Rosal, Pontevedra	41.92	-8.83	45	23
Rosaldi	PRF-068	Lleida, Lleida	41.63	0.58	205	23
Ruixou - 1	PRF-077	La Torre de Cabdella, Lleida	42.33	0.98	840	61
Sant Esteve - 1	PRF-125	Sant Esteve Sesrovires, Barcelona	41.50	1.87	190	23
Sant Jaume	PRF-118	Olot, Lleida	42.15	2.55	560	40
Sant Sadurní - 1	PRF-126	Sant Sadurní d'Anoia, Barcelona	41.42	1.78	160	23
Santiaguera	PRF-056	Huesa del común, Teruel	41.03	-0.93	800	23
Saramaña	PRF-128	Soleràs, Lleida	41.40	0.68	400	23

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Table 1. Continued.

Accession name ^z	Accession code	Site of collection in Spain	Latitude	Longitude	Elevation (m)	Collecting source ^y
Seremeña	PRF-129	Els Torms, Lleida	41.38	0.72	480	23
Seremeñera	PRF-043	Mollerusa, Lleida	41.63	0.88	250	62
Sermenyer	PRF-073	Almenar, Lleida	41.77	0.62	270	23
Sermeña	PRF-069	Almenar, Lleida	41.78	0.57	285	23
Sermeñeta	PRF-052	Seròs, Lleida	41.48	0.40	130	62
Sermeñeta	PRF-053	Seròs, Lleida	41.48	0.40	130	62
Sopeira - 1	PRF-094	Sopeira, Huesca	42.27	0.73	670	62
Sopeira - 2	PRF-095	Sopeira, Huesca	42.27	0.73	670	62
<i>Sopeira - 3</i>	PRF-096	Sopeira, Huesca	42.27	0.73	675	62
<i>Tardia Fuenmayor - 1</i>	PRF-013	Fuenmayor, La Rioja	42.45	-2.55	435	62
<i>Tardia Fuenmayor - 2</i>	PRF-014	Fuenmayor, La Rioja	42.45	-2.55	435	62
Tauñl - 1	PRF-100	La Vall de Boí, Lleida	42.52	0.85	1500	23
Temprana Lardero	PRF-001	Lardero, La Rioja	42.42	-2.45	435	23
Temprana Torrecilla	PRF-002	Lardero, La Rioja	42.43	-2.45	440	23
Tendral	PRF-131	Castelldans, Lleida	41.52	0.77	235	23
Tendral antigua	PRF-074	Almenar, Lleida	41.77	0.62	270	23
Urriaga Lardero	PRF-016	Lardero, La Rioja	42.43	-2.45	410	23
<i>Vallcalent - 1</i>	PRF-080	Vallcalent, Lleida	41.62	0.60	185	25
Vallcanera - 1	PRF-138	Vallcanera - Sils, Girona	41.83	2.73	95	23
Verdilla Chata	PRF-057	Huesa del común, Teruel	41.03	-0.93	800	23
<i>Viella - 1</i>	PRF-109	Vielha e Mijaran, Lleida	42.70	0.78	1040	23
<i>Viella - 2</i>	PRF-110	Vielha e Mijaran, Lleida	42.70	0.78	1040	23
<i>Vilasana-1</i>	PRF-136	Vilasana, Lleida	41.65	0.92	235	23
Villaviciosa - 1	PRF-022	Villaviciosa, Asturias	43.48	-5.42	10	23
<i>Villaviciosa - 2</i>	PRF-025	Villaviciosa, Asturias	43.48	-5.42	10	23
<i>Villaviciosa - 3</i>	PRF-026	Villaviciosa, Asturias	43.48	-5.42	10	23
<i>Villaviciosa - 4</i>	PRF-027	Villaviciosa, Asturias	43.48	-5.42	10	23
<i>Vimianzo - 1</i>	PRF-035	Vimianzo, La Coruña	43.10	-9.02	140	23
Viñalets	PRF-041	Lleida, Lleida	41.62	0.70	180	62
Xata	PRF-119	Olot, Lleida	42.15	2.55	560	40
Reference cultivars						
Abbe Fetel	REF-001	Universidad Pública de Navarra Germplasm Bank				
Blanquilla	REF-002	Universidad Pública de Navarra Germplasm Bank				
Bonne Louise d'Avranches	REF-003	Universidad Pública de Navarra Germplasm Bank				
Castell	REF-004	ETSIA-UdL Germplasm Bank				
Conference	REF-005	Universidad Pública de Navarra Germplasm Bank				
D. Comice	REF-006	Universidad Pública de Navarra Germplasm Bank				
Flor de Invierno	REF-007	Universidad Pública de Navarra Germplasm Bank				
Grand Champion	REF-008	ETSIA-UdL Germplasm Bank				
Limonera (Dr. Jules Guyot)	REF-009	Universidad Pública de Navarra Germplasm Bank				
Magallona	REF-010	ETSIA-UdL Germplasm Bank				
Roma	REF-011	ETSIA-UdL Germplasm Bank				
Supercomice	REF-012	Universidad Pública de Navarra Germplasm Bank				
Williams B.C. (Bartlett)	REF-013	Universidad Pública de Navarra Germplasm Bank				

^zAccessions in italics were named after the village in which they were collected.

^yFAO/IPGRI (2001) codes for collecting source: 12 = shrubland, 21 = field, 23 = backyard or home garden, 25 = pasture, 28 = park, 40 = institute, 61 = roadside, 62 = road margin.

european pear (*P. communis*) (Fernández-Fernández et al., 2006; Yamamoto et al., 2002a, 2002b), SSR derived from *Malus* and *Pyrus* have been used to reveal pear genetic diversity among pear cultivars (Bao et al., 2007; Bassil et al., 2008, 2009; Brini et al., 2008; Ghosh et al., 2006; Jiang et al., 2009; Katayama et al., 2007; Kimura et al., 2002; Sisko et al., 2009; Volk et al., 2006; Wünsch and Hormaza, 2007; Xuan, 2008).

The present study aims to determine the genetic identity of the *Pyrus* accessions curated at the ETSIA-UdL Germplasm Bank, to estimate the genetic diversity of the collection, to identify the genetic structure and relationships among its

accessions, and to establish a representative core collection, using a set of highly informative SSR markers, to optimize the conservation and use of this germplasm.

Materials and Methods

PLANT MATERIAL. One hundred forty-one pear accessions, collected from 12 different provinces in northern Spain, were used for this study. The plant material is maintained in the pear collection of the ETSIA-UdL Germplasm Bank. The collection is composed of old and local pear cultivars, and the name and

origin of each accession is given in Table 1. All the accessions were prospected from singular trees that at the moment of their collection were actively cultivated (in backyards or small farms) or were abandoned old trees. When the accession was prospected in an abandoned spot and its local denomination could not be known, it was named after the village where it was obtained (indicated by italics in Table 1). All of the accessions have been characterized phenotypically in an earlier work (Urbina et al., 2007).

An additional set of 13 well-known pear cultivars was obtained from the pear collections of ETSIA-UdL and Universidad Pública de Navarra (UPNA, Pamplona, Spain) and has been included in the analysis as a reference. Reference cultivars were chosen among those currently grown in Spain and aiming, at the same time, to represent a wide variability and genetic diversity (Wünsch and Hormaza, 2007). The commercial importance of these cultivars is, nevertheless, very different: six cultivars (Abbe Fetel, Blanquilla, Conference, Comice, Limonera, and ‘Williams’) account for >80% of pear production in Spain and ≈70% in western Europe (World Apple and Pear Association, 2009); four cultivars (Flor de Invierno, Castell, Roma, and Magallona) are old cultivars that remain important in Spain at a regional level, and the remaining three cultivars (Bonne Louise, Grand Champion, and Supercomice) are used mainly as pollenizers or are cultivated to some extent in a few regions.

SSR ANALYSIS. Total genomic DNA from all pear cultivars included in Table 1 was extracted from young leaves according to the Fulton et al. (1995) protocol. Extracted DNA was quantified in a spectrophotometer (Biomate-3; Thermo Spectronic, Rochester, NY). Ten SSR primer pairs developed from european and asian pear (Yamamoto et al., 2002c), and four developed from apple (Gianfranceschi et al., 1998) were used for the initial assay, and eight were chosen after gel clarity and reliability of alleles (Table 2). Polymerase chain reactions (PCR) were performed in a final volume of 15 µL containing 75 ng of genomic DNA, 0.2 µM primer, 0.2 mM of each dNTPs, 1.5 mM MgCl₂, 1× reaction buffer, and 1.0 units of Taq polymerase (Biotools, Madrid, Spain). Reactions were carried out in a thermal cycler (model 2720; Applied Biosystems, Foster City, CA) with the following temperature profile: an initial 2.5-min denaturation step at 94 °C, followed by 30 cycles

of 30 s at 94 °C, 1 min at annealing temperature and 1 min at 72 °C, and a final 5-min extension step at 72 °C. The annealing temperature used was 50 °C for all loci except for NB103a (42 °C) and NB106a and RLG-1 (47 °C). PCR products were separated and detected on an acrylamide gel using a Sequi-Gen GT (Bio-Rad, Hercules, CA) sequencing unit, and they were visualized by silver staining (Bassam et al., 1991). A 30- to 330-bp AFLP DNA ladder (Invitrogen Life Technologies, Barcelona, Spain) was used as the molecular size standard. The bands of amplified DNA were scored manually.

DIVERSITY ANALYSIS. All unambiguous fragments corresponding to SSR markers were scored for the presence (1) and absence (0) of each band. The genetic information was assessed using the following parameters: number of alleles per locus (A), effective number of alleles [$A_e = (\sum p_i^2)^{-1}$, where p_i is the frequency of the i^{th} allele], number of rare alleles per locus (B = no of alleles with frequency < 0.05), observed heterozygosity (H_o , direct count), expected heterozygosity ($H_e = 1 - \sum p_i^2$), and Nei’s diversity index (DI) (Nei, 1987). Discrimination power [PD (Tessier et al., 1999)] was also calculated according to the latter formula, where p_i represents the frequency of the i^{th} genotype.

ANALYSIS OF GENETIC STRUCTURE. To assess the structure of the genetic diversity within the collection, we used three approaches: graphic clustering from similarity data, Bayesian clustering, and factorial correspondence analysis (FCA). The genetic similarity between accessions was calculated with presence/absence data for each accession according to Nei and Li (1979) and the unweighted pair group method with arithmetic mean (UPGMA) clustering with Phylip 3.65 (Felsenstein, 1989). Bootstrap analysis was performed with 5000 replicates. We also used the Bayesian model-based clustering procedure implemented in STRUCTURE 2.2 (Pritchard et al., 2000). We used the admixture model and 250,000 iterations were computed following a 75,000 iteration burn-in period. Each Markov chain was run 10 times for k (the assumed number of groups) varying from one to 10. We used the Δk method described by Evanno et al. (2005) to examine the rate of change in successive posterior probabilities over a range of k values and to estimate the appropriate k value. A genotype was assigned to the group for which it had the highest membership coefficient. Modal assignment across all replicate runs was used to determine the final placement of a genotype in a group, and the results were visualized in a barplot using Distruct (Rosenberg, 2004). Finally, multivariate analyses were performed with GENETIX 4.05 software (Belkhir et al., 2004) by FCA. Rare alleles (frequency below 2%) were eliminated and considered as missing data because they may bias analyses (Breton et al., 2008).

The genetic structure of the collection was further investigated by a hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN 3.11 (Excoffier et al., 2005). The significance of the partitioning of genetic variance among groups was tested. Groups were defined according to clusters obtained by the Bayesian analysis. Descriptive statistics, including variation between clusters (F_{ST}) and diversity within groups including Nei’s DI, number of polymorphic alleles, and

Table 2. Microsatellite code, linkage group, size range, number of alleles per locus (A), number of effective alleles per locus (A_e), observed (H_o) and expected (H_e) heterozygosity, Nei diversity index (DI), and discriminant power (P_D) of eight SSR loci analyzed in 126 unique european pear accessions of the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) pear collection.

Locus ^z	Linkage group no.	Size range (bp)	A	A_e	H_o	H_e	DI	P_D
CH01h10	8	102–126	10	6.04	0.70	0.83	0.84	0.94
NB103a	5	82–138	15	6.37	0.76	0.84	0.85	0.95
NB105a	11	144–186	11	6.91	0.89	0.86	0.86	0.96
NB106a	9	84–134	14	6.42	0.75	0.84	0.85	0.94
NB109a	3	128–206	15	9.34	0.91	0.89	0.90	0.97
NH023a	3	120–174	12	2.89	0.51	0.65	0.66	0.81
NH029a	9	86–106	9	5.96	0.87	0.83	0.84	0.94
RLG1–1	1	196–238	11	6.97	0.58	0.86	0.86	0.94
Total			97	51.41				
Avg			12.13	6.36	0.74	0.83	0.83	0.93

^zPrimer pairs developed by Yamamoto et al. (2002c), except for CH01h10, which was developed by Gianfranceschi et al. (1998).

allelic richness (El Mousadik and Petit, 1996), were estimated using ARLEQUIN and FSTAT (Goudet, 1995).

CONSTRUCTION OF THE CORE COLLECTION. The number and selection of accessions for inclusion in the core set was identified using the MSTRAT software (Gouesnard et al., 2001), based upon the maximization (M-strategy) of the number of alleles retained in each locus (Schoen and Brown, 1995). We used Nei's DI (Nei, 1987) as a second maximization approach. Optimal core size was identified using the feature of MSTRAT that measures and plots the fraction of total diversity retained in cores of increasing size. We performed 20 independent sampling runs and identified the inflection point on the resulting mean curvilinear plot as the optimal core size (Gouesnard et al., 2001). Once the appropriate core size was identified, we examined 20 possible core sets and developed a consensus set retaining the most commonly found accessions among the 20 replicate core sets. The representativeness of the core collection was validated according to the following criteria (modified from Brown, 1989, Grenier et al., 2000): 1) recovery of all the alleles present in the whole collection, 2) no significant differences in the frequency distribution of frequent alleles in at least 95% of loci between the core and the whole collection (evaluated with chi-square), and 3) no significant differences in variability parameters (H_o and H_e) between the core subset and the entire collection (evaluated by Friedman's repeated measures analysis). All the comparisons were carried out with SPSS (version 15.0; SPSS, Chicago).

Results and Discussion

SSR POLYMORPHISM. The eight SSR loci amplified a total of 97 alleles in the 154 pear cultivars analyzed (141 local accessions and 13 reference cultivars), varying from nine (NH029a) to 15 (NB109a) alleles per locus, with an average of 12.13 alleles per locus, and the effective number of alleles being 6.43 (Table 2). H_o ranged from 0.51 (NH023a) to 0.91 (NB109a), with an average of 0.74, whereas H_e ranged from 0.65 (NH023a) to 0.89 (NB109a), with an average of 0.83. Nei's DI was high (>0.8) for all SSR loci except for NH023a (DI = 0.66). The mean estimate of power of discrimination (P_D) was relatively high, with an average value of 0.93, and ranged from 0.64 for NH023a to 0.97 for NB109a. All accessions, except 16, had at least one of the 48 rare alleles identified. Accessions that contributed most in terms of rare alleles were 'Sant Sadurni-1' and 'Rengá', with seven rare alleles each. A total of seven unique alleles (alleles present in only one accession) was found in six accessions (Table 3).

Reliable genetic markers are essential for efficient cultivar identification and the establishment of genetic relationships among them. The eight SSR markers used in this study have proven their reliability in assessing identities and diversity retained within the ETSIA-UdL pear germplasm collection. The overall allelic diversity displayed by the eight SSR loci has revealed a high genetic variation in the pear germplasm included in this study. The results for allelic number per locus fall within the range of values reported for SSR studies of cultivated *P. communis* genotypes (Bassil et al., 2008, 2009; Brini et al., 2008; Sisko et al., 2009; Wünsch and Hormaza, 2007, Xuan, 2008). H_e summarizes the fundamental genetic variation of a population or species in a single parameter (Berg and Hamrick, 1997) and, for that reason, is a commonly used genetic DI that allows comparisons with the literature. The

average H_e found in our study ($H_e = 0.83$) is slightly higher than values reported by other studies of variation in *P. communis* germplasm performed with different sets of SSRs and fewer cultivars (Bassil et al., 2008, 2009; Brini et al., 2008; Sisko et al., 2009; Wünsch and Hormaza, 2007, Xuan, 2008).

CULTIVAR IDENTIFICATION. Seven groups of synonyms, including 22 accessions as a whole, were identified (Table 4). Some were expected because the accessions received very similar or identical denominations, such as the identity groups of 'Limonera', 'Roma', and 'Blanquilla' types. However, other groups of synonymies are composed of accessions that have different names (or were unknown) and geographical origin, probably indicating the spread of selected plants through grafting. On the other hand, several homonyms have also been shown: both 'Pera d'Hivern' accessions (PRF-078 and PRF-085) differed at the eight loci and shared only four alleles. The same situation was observed for both 'Tendral' accessions (PRF-131 and PRF-074), and one of the 'Limoneras' (PRF-137) that shared only six alleles with the rest of them. The accession called 'Blanquilla Durró' differs from the rest of 'Blanquillas', but it is probably a descendant of unknown pedigree, as it shares one allele with 'Blanquilla' on each of the eight loci analyzed. Finally, slight differences (just one allele at NB105a loci) were detected between the two accessions named

Table 3. Unique simple sequence repeat alleles in the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) pear collection.

Locus	Allele (bp)	Accession name (code)
NB103a	138	Sant Jaume (PRF-118) ²
NB105a	150	Pera d'Aigua (PRF-120)
NB106a	114	Donguindo Venta Lardero (PRF-009)
NH023a	136	Taüll-1 (PRF-100)
	140	Peñon (PRF-023)
	148	Donguindo Venta Lardero (PRF-009)
NH029a	106	Sopeira-2 (PRF-095)

²Numbers in parentheses correspond to the accession codes listed in Table 1.

Table 4. Synonyms identified in the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) pear collection by SSR fingerprinting.

Identity group	Synonyms [name (code)] ²
1	Flor de invierno (REF-007), Flor de Invierno (PRF-064), Piedrahita-2 (PRF-076)
2	Villaviciosa-2 (PRF-025), Villaviciosa-4 (PRF-027)
3	Limonera (REF-009), Limonera francesa (PRF-019), Limonera Mutalbatarrac (PRF-020)
4	Roma (REF-011), Roma Acedas (PRF-010), Roma Arbaca-1 (PRF-045)
5	Pela de Roure (PRF-065), Balaguer Flix-5 (PRF-050), La Guardia-1 (PRF-084), Pera d'Hivern (PRF-085), De Invierno (PRF-111), Coure (PRF-116)
6	Blanquilla (REF-002), Blanquilla Rosada (PRF-058)
7	Donguindo Venta Lardero (PRF-009), Donguindo Fuenmayor (PRF-012), Cristobal de Bejar-1 (PRF-039)

²Numbers in parentheses correspond to the accession codes listed in Table 1.

‘Azúcar Verde’. This minor difference can be due to some somatic mutation that it is known to occur in long-lived trees that have been vegetatively propagated by grafting. All in all, 15 genotypes that were identified as ‘synonyms’ were not considered further in the analysis of genetic diversity, which was therefore restricted to the remaining 126.

GENETIC RELATIONSHIPS AMONG CULTIVARS. Pairwise Nei and Li genetic similarity coefficients ranged from 0.057 to 0.938. Except for ‘Magallon’ and ‘Castell’, the commercial cultivars included as a reference were arranged in the upper half of the dendrogram (Fig. 1A) based on UPGMA analysis. Several small clusters, strongly supported by bootstrap values >70%, were arranged around the reference cultivars Roma, Blanquilla, Magallon, Williams, Bonne Louise, and Flor de Invierno. The accessions within those clusters were also closely similar to the reference cultivars in their morphological traits (Urbina et al., 2007). Other clusters with strong bootstrap support and morphological similarities included the ancient cultivars Temprana de Julio, Lombardia, Tendral, De agua, Azúcar verde, and Sermeñeta.

ANALYSIS OF GENETIC STRUCTURE. Multivariate analysis (FCA) computed on all the accessions and reference cultivars displayed three partially overlapping groups (Fig. 2). Accessions of ‘Roma’ and ‘Blanquilla’ types clustered together and isolated from the others (Cluster A in Fig. 1). There was a main cluster (B) composed mostly of the same accessions that appeared in the lower half of UPGMA dendrogram, along with ‘Magallon’ and ‘Castell’. The third cluster (C) grouped the remaining reference cultivars with accessions mostly placed in the upper third of the dendrogram. With the 126 unique accessions and 13 reference cultivars used in this study, the most probable value of k for Bayesian analysis was $k = 3$. Among the subsequent 10 separate MCMC chains run with $k = 3$, individual assignments to groups were highly correlated (>0.95) among runs. The affinity of most individuals (80%) to their assigned groups was strong, as their respective Q membership probabilities were >0.8 (Breton et al., 2008). In the remaining 29 accessions with lower affinities ($0.42 \leq Q < 0.80$), their placement in a group reflects consistent assignments among runs. The three groups are represented with different colors in Fig. 1B. Group 1 (black) contains 34 accessions and nine reference cultivars, with the majority belonging to cluster C observed in FCA analysis. Group 2 (white) is composed of 18 individuals, including all ‘Roma’ and ‘Blanquilla’-like accessions, and several

Fig. 1. Genetic relationships among the 126 unique pear accessions of the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) pear collection and 13 cultivars used as a reference (marked with an asterisk preceding their name). (A) Dendrogram based on Nei and Li similarity matrix and UPGMA clustering. Bootstrap values >70% are placed on branches. (B) Group probabilities obtained for $k = 3$ by Bayesian clustering. Each bar represents the genetic background of an individual according to the proportion derived from each of the three different groups. Color codes for each genetic group are: group 1 = black, group 2 = white, group 3 = gray.

Fig. 2. Factorial correspondence analysis (FCA) based on polymorphism at eight SSR loci for 139 individuals [126 accessions from the Escuela Técnica Superior de Ingeniería Agraria - Universidad de Lleida (ETSIA-UdL) pear collection and 13 reference cultivars]. Accession colors reflect the consistent assignment using Bayesian analysis to one of the groups defined in Fig. 1B.

accessions in admixture mostly with group 1. The third group (gray) comprises the remaining 76 accessions along with ‘Magallon’ and ‘Castell’ reference cultivars. Though there was not a straightforward correspondence between the geographic origin of the accessions and their group placement, which agrees to the traditional exchanges of plant material through grafting in the Ebro Valley provinces (La Rioja, Huesca, Teruel, and Lleida), certain grouping trends could be observed for some origins. Thus, most of the accessions with Atlantic origin (those collected in La Coruña, Pontevedra, Asturias, and Cantabria) were clustered in group 1, and most of the Mediterranean accessions collected in coastal provinces (Barcelona and Girona) were assigned to group 3.

Based on AMOVA analysis, significant variance differences were found among the three groups identified with Bayesian clustering. The overall F_{ST} value of 0.074 suggested a moderate but highly significant ($P < 0.0001$) differentiation between groups. The highest proportion of allele variability was due to

differences among accessions (92.6% of total variation in AMOVA analysis). Genetic diversity indexes were calculated by group (Table 5). Nei’s gene diversity varied from 0.74 to 0.83, revealing a high proportion of heterozygous individuals in the three groups. However, important differences in allelic composition and richness were observed among groups. The number of alleles represented in groups 1 and 2 for each of the eight SSR was around 40% to 70% of the total, whereas group 3 represented all the alleles except for two. Regarding the alleles present in only one group (private alleles), 23 of the 24 identified were found in group 3. Moreover, the seven accessions with unique alleles (Table 3) were included within this group. Because the higher allelic diversity observed in group 3 could be due to its larger size, we calculated their allelic richness (El Mousadik and Petit, 1996) to properly evaluate allelic diversity among groups, as this rarefaction method performs scaling to the smallest group ($N = 18$ in this study) to compensate for differences in size. The higher diversity observed in group 3 was

Table 5. Descriptive information for each of the three groups of genotypes identified by Bayesian clustering analyses. Summary statistics are given for each group as a whole. Summary statistics include the number of individuals in each group (N), Nei’s genetic diversity, the number of alleles exclusive to the identified clusters, the number of unique alleles identified in the group, and the total number of alleles scored across all microsatellite loci. Allelic richness is scaled to the smallest group.

Group no.	N	Gene diversity (Nei)	Alleles in the group (no.)			Alleles at each SSR locus (no.)								Allelic richness
			Total	Exclusive ^z	Unique ^y	CH01h10	NB103a	NB105a	NB106a	NB109a	NH023a	NH029a	RLG1-1	
1	43	0.75	60	0	0	8	7	9	5	11	5	8	7	6.64
2	18	0.74	50	1	0	6	6	6	8	7	3	6	8	6.25
3	78	0.83	95	23	7	10	15	10	13	15	12	9	11	9.13

^zIdentified in only one group by Bayesian analysis.

^yIdentified in only one accession.

Fig. 3. Relationship between the number of individuals in the core set and the allelic diversity (%) retained.

confirmed, as it showed (Table 3) much higher allelic richness than the others.

The results obtained through the different approaches used to analyze the genetic structure of the collection were coherent, revealing three groups with moderate, but significant, differentiation. The identification of private alleles, the clustering of all accessions with unique alleles in only one group, and remarkable differences in allelic richness among groups constitute further evidence of the uniqueness of this material. It is remarkable that most of the cultivars included as a reference clustered together in group 1 in spite of being heterogeneous material from different origins. In fact, the four reference cultivars clustered in groups 2 and 3 (Blanquilla, Roma, Castell, and Magallon) are ancient southern European cultivars grown in Spain since at least the 18th and 19th centuries. Those four cultivars had already been described as very different from a diverse group of more than 50 cultivars, with European and American origins that include old cultivars and others derived from them in recent breeding programs (Wünsch and Hormaza, 2007).

CORE COLLECTION. The relationship between sample size and allelic diversity (Fig. 3) showed that >99% of the diversity could be retained with a 35-member core set. The consensus set of accessions identified for the ETSIA-UdL pear core collection are indicated in bold in Table 1. The total number of alleles recovered in the core, and H_e and H_o , were similar ($P > 0.05$) to those of the whole collection ($H_{o-UdL} = 0.734 \pm 0.151$, $H_{o-core} = 0.735 \pm 0.076$; $H_{e-UdL} = 0.828 \pm 0.076$, $H_{e-core} = 0.850 \pm 0.054$). Regarding the allele frequencies, the chi-squared tests showed that the core subset had a similar ($P > 0.05$) allelic distribution for all eight loci. As a whole, the accessions selected for the core set constitute a representative sample of the diversity retained in the ETSIA-UdL collection. The maximization strategy has narrowed the collection to a smaller representative sample with a minimum of redundancy, constituting an efficient and accessible entry point in the ETSIA-UdL pear collection for breeding and research communities.

Our results highlight that the overall genetic diversity represented in currently cultivated pears is only a small fraction

of that which is existent within the species and that the germplasm curated at the ETSIA-UdL collection is a good instance of genetic distinctness with respect to the main pear cultivars used in European orchards. Therefore, the genetic variability found among ancient Spanish pear cultivars needs to be further studied and preserved as a potential source of desirable traits for the modern pear growing to optimize breeding progress.

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