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Genetic diversity and structure of local apple cultivars from Northeastern Spain assessed by microsatellite markers

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Abstract A set of 493 old and local Spanish accessions of apple (*Malus* x *domestica* Borkh) maintained at three collections in Northeastern Spain was studied using 16 simple sequence repeats in order to estimate their genetic diversity and to identify the genetic structure and relationships among their accessions. An additional diverse set of 45 apple cultivars, including old Spanish and international cultivars, was added as reference. Genetic analyses performed by Bayesian model-based clustering revealed a very strong differentiation of two major groups. The first one clustered 159 individuals (52 % of unique genotypes) including local accessions and six old Spanish cultivars. The second major group was formed by 145 individuals, including 38 international reference cultivars and one old Spanish cultivar. Nested Bayesian clustering was applied to those two groups and two and four sub-groups were found at each one, respectively. The identification of private and unique alleles, and the remarkable differences in allelic richness among groups and sub-groups constitute further evidence of a clear genetic structure. The results obtained through the factorial correspondence and analyses of molecular variance confirmed those obtained by Bayesian analyses, revealing moderate but significant differentiation among the two major groups (F_{ST}=0.076) and the six sub-groups (F_{ST}= 0.111). Our results highlight that the genetic diversity encompassed by currently cultivated apple accounts only for a small fraction of that existing within the species, and that an important part (≈60 %) of the local material analyzed constitutes a good example of genetic distinctness with respect to the main cultivars used in European orchards.

Keywords *Malus* x *domestica* · SSR · diversity · genetic structure · Northeastern Spain

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Introduction

Cultivated apple (*Malus* x *domestica* Borkh.) is one of the most important fruit crops grown in temperate zones worldwide (Janick and Moore 1996) and the most important fruit crop of the Rosaceae family in the world (Food and Agriculture Organization of the United Nations 2011) and also in Spain. The genetic base of the domesticated apple has been eroded dramatically in the last centuries (Hokanson et al. 2001), as most cultivars bred during the twentieth century were obtained from a reduced number of progenitor lineages and, therefore, share a high degree of identity (Noiton and Alspach 1996). The narrowness of this genetic base has been further eroded since nowadays, despite apple production areas being widespread geographically, production is based on a reduced number of cultivars. This trend toward genetic uniformity in commercial apple orchards has been further accentuated by the release of additional mutants of popular cultivars (Brooks and Olmo 1991, 1994). "Golden Delicious", "Gala", "Red Delicious", and "Idared" cultivars and their mutants account for 48 % of the European Union production in 2010 and for 85 % of the Spanish production (World Apple and Pear Association 2010). As a consequence, many of the traditional or locally well-adapted 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 Spain, there are currently seven apple collections integrated in the Network of Collections of the National Program of Plant Genetic Resources (INIA 2011). Since collection holders in the country operated relatively independently from each other, the diversity conserved on these collections at the supra-collection level is largely unknown. In order to achieve a more accurate knowledge of apple genetic resources conserved in Northeastern Spain, three collections, namely those at Public University of Navarra (UPNA, Pamplona), University of Lleida (UdL, Lleida), and Estación Experimental Aula Dei-CSIC (EEAD-CSIC, Zaragoza), have been established during the last years' several coordinated actions. These collaborations have already helped to establish standardized methodologies to undertake the process of morpho-agronomic and molecular characterization (Urrestarazu et al. 2010, 2011).

Within apple collections, microsatellite markers have been favored over others to establish unique genetic identities or fingerprints and to assess genetic diversity (Garkava-Gustavsson et al. 2008; Gharghani et al. 2009; Gianfranceschi et al. 1998; Guarino et al. 2006; Guilford et al. 1997; Hokanson et al. 1998; Liebhard et al. 2002; Pereira-Lorenzo et al. 2007) due to their high polymorphism level, reproducibility, and relative ease of analysis (Schlötterer 2004). As a great number of simple sequence repeat (SSR) are available, it was very common that each institution used different sets of markers to characterize their accessions, which makes difficult to compare and evaluate the diversity among collections. For that reason, the European Cooperative Programme for Plant Genetic Resources (ECPGR) has published several lists of recommended markers (Evans et al. 2007; Laurens et al. 2004). Those lists have been modified over the years, as more information about polymorphism and linkage was available (Kenis and Keulemans 2005; Silfverberg-Dilworth et al. 2006; Velasco et al. 2010). Nowadays, the ECPGR list includes 17 SSRs that span most of the apple genome (Evans et al. 2007), and have been tested on a set of standard *Malus* accessions.

Moreover, the characterization of accessions using a common set of SSRs also allows clarifying the genetic relationships between them, which permits an improved management of genetic resources. Bayesian inference has been proposed in the last years as a suitable tool to analyze population structure (Pritchard et al. 2000) and it is widely used in conservation biology to quantify relationships and differences among populations and to detect hybridization (Breton et al. 2008). This method reveals introgression even if the parental populations cannot be sampled and it can assign individuals to populations without requiring previous information (Iketani et al. 2010). Therefore, it is an effective method to analyze human-disturbed materials (e.g. cultivars), since the assemblage of these materials cannot be strictly regarded as biological populations even if collected within a small area. As a consequence, Bayesian inference has become a powerful tool to assess the genetic structure in tree species such as pear (Ferreira dos Santos et al. 2011; Iketani et al. 2010; Miranda et al. 2010; Volk et al. 2006), plum (Horvath et al. 2011), sweet cherry (Mariette et al. 2010), olive (Breton et al. 2008; Erre et al. 2010) and chestnut (Pereira-Lorenzo et al. 2010). In apple, it has been mainly used to reveal genetic structure and relationships in wild *Malus* species such as *Malus sieversii* (Richards et al. 2009), *Malus orientalis* (Volk et al. 2009) and *Malus sylvestris* (Coart et al. 2003; Larsen et al. 2006), but studies evaluating structure within cultivated apple material remain scarce (Pereira-Lorenzo et al. 2008).

The present study aims to determine the genetic identity of the *Malus* accessions curated at three Northeastern Spanish collections, to identify their genetic structure, and to establish the relationships among these accessions and a diverse set of international reference cultivars.

Material and methods

Plant material

A total of 493 accessions maintained at the Germplasm Banks of UPNA (322 accessions), UdL (104 accessions), and EEAD-CSIC (67 accessions) in Northeastern Spain were used in this study (Electronic supplementary material (ESM) 1 and 2). The three collections are composed of old and local apple cultivars, prospected from singular trees that, at the moment of their collection, were actively cultivated (in backyards or small farms) or abandoned old trees. When an accession was prospected at an abandoned spot and its local denomination could not be learnt, it was named after the village where it was collected (indicated by italics in ESM 1). The accessions maintained in the collections were gathered after several inventory and collection missions at 22 Spanish provinces, mainly in the Northeastern part of the country (Navarre, Basque Country, La Rioja, Aragon, and Catalonia regions). In addition, 45 reference cultivars were included in this study (Table 1). Several kinds of references were included: well-known old Spanish cultivars, international cultivars introduced, and cultivated at least to some extent in Spain since the 1950s, and international cultivars (modern and old) widely diverse in terms of origin and parentage.

SSR analysis

Newly expanded leaves of each accession were collected, inmediately frozen in liquid nitrogen and stored at −80°C until further processing. Leaf samples were ground to a fine powder using liquid nitrogen and tungsten beads in a micro-dismembrator (B. Braun Biotech International, Melsungen, Germany). Genomic DNA was isolated from 50 mg of this

Table 1 Apple cultivars used as reference in this study, indicating reported parentage and group placement by Structure analysis

N	Cultivar name	Type[a]	Reported parentage	Group[b]
1	**Esperiega**	**OS**	**Unknown**	**1.1**
2	**Manyaga**	**OS**	**Unknown**	**1.1**
3	**Verde Doncella**	**OS**	**Unknown**	**1.1**
4	**Ortell**	**OS**	**Unknown**	**1.1**
5	Camuesa	OS	Unknown	1.1
6	**Geza Miña**	**OS**	**Unknown**	**1.2**
7	**Democrat**	**MI**	**Hoover×Unknown**	**2.1**
8	Cox's Orange Pippin	OI	Ribston Pippin seedling	2.1
9	JerseyMac	MI	NJ24×July Red	2.1
10	**Rome Beauty**	**OI**	**Unknown**	**2.2**
11	**Delcon**	**MI**	**Delicious×Jonathan×Ben Davis**	**2.2**
12	**Fuji**	**MI**	**Ralls Janet×Delicious**	**2.2**
13	**Fukutami**	**MI**	**Jonathan×Ralls Janet**	**2.2**
14	**Gloster**	**MI**	**Weisser Winterglockenapfel×Delicious**	**2.2**
15	**Jonadel**	**MI**	**Jonathan×Delicious**	**2.2**
16	**Jonathan**	**OI**	**Unknown**	**2.2**
17	**Melrose**	**MI**	**Jonathan×Delicious**	**2.2**
18	**Red Delicious**	**OI**	**Unknown**	**2.2**
19	**Reinette Blanche**	**OI**	**Unknown**	**2.2**
20	**Stayman Winesap**	**OI**	**Unknown**	**2.2**
21	**Turley Winesap**	**MI**	**Stayman Winesap OP**	**2.2**
22	Bost Kantoi	OS	Unknown	2.2
23	Florina	MI	PRI 612-1×Jonathan	2.2
24	Akane	MI	Jonathan×Worcester Pearmain	2.2
25	Granny Smith	MI	Unknown	2.2
26	Idared	MI	Jonathan×Wagenerapfel	2.2
27	Kidd's Orange Red	MI	Delicious×Cox's Orange Pippin	2.2
28	Reine des Reinettes	OI	Unknown	2.2
29	Spartan	MI	McIntosh×Newtown Pippin	2.2
30	**Golden Delicious**	**OI**	**Unknown**	**2.3**
31	**Charden**	**MI**	**Golden Delicious×Reinette Clochard**	**2.3**
32	Delbard Estivale	MI	Golden Delicious cross	2.3
33	Freyberg	MI	Golden Delicious×Cox's Orange Pippin	2.3
34	Gala	MI	Kidd's Orange Red×Golden Delicious	2.3
35	Golden Supreme	MI	Chance seedling of G. Delicious	2.3
36	Kinsei	MI	Golden Delicious×Ralls Janet	2.3
37	Mutsu	MI	Golden Delicious×Indo	2.3
38	Ozark Gold	MI	A1291×Golden Delicious	2.3
39	Telamon	MI	Wijcik×Golden Delicious	2.3
40	Trajan	MI	Golden Delicious×Wijcik	2.3
41	Tuscan	MI	Wijcik×Greensleeves	2.3
42	**Red Boskoop**	**MI**	**Unknown**	**2.4**
43	Gravenstein	MI	Unknown	2.4
44	McIntosh	OI	Unknown	2.4
45	Prima	MI	PRI 14-510×N.J. 123249	2.U

[a]*OS* Old Spanish cultivar, *OI* International cultivars grown in Spain since at least 1950s, *MI* modern international cultivars

[b]Group assignment by Structure analysis, cultivars in bold have $qI>0.8$ to the assigned group

fine powder with Qiagen Dneasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration of each sample was determined using a NanoDrop 2000 (Thermo Fischer Scientific, Wilmington, DE, USA), and DNA working dilutions of each sample were adjusted to 5 ng μl^{-1}.

Table 2 Microsatellite code, linkage group, PCR details, and size range (base pair) of 16 SSR loci analyzed in 304 unique accessions

Locus	Linkage group number	Multiplex	Dye	Size range (bp)	Forward primer sequence 5′→3′	Reverse primer sequence 5′→3′
Hi02c07[a]	1	C	VIC	103–151	AGAGCTACGGGGATCCAAAT	GTTTAAGCATCCCGATTGAAAGG
CH02c06[b]	2	C	PET	203–266	TGACGAAATCCACTACTAATGCA	GATTGCGCGCTTTTTAACAT
GD12[c]	3	C	NED	140–191	TTGAGGTGTTTCTCCCATTGGA	CTAACGAAGCCGCCATTTCTTT
CH05f06[b]	5	B	NED	165–191	TTAGATCCGGTCACTCTCCACT	TGGAGGAAGACGAAGAAGAAAG
CH03d07[b]	6	C	VIC	181–227	CAAATCAATGCAAAACTGTCA	GGCTTCTGGCCATGATTTTA
CH04e05[b]	7	A	PET	177–229	AGGCTAACAGAAATGTGGTTTG	ATGGCTCCTATTGCCATCAT
CH01h10[b]	8	A	PET	89–135	TGCAAAGATAGGTAGATATATGCCA	AGGAGGGATTGTTTGTGCAC
CH01h02[b]	9	A	NED	230–254	AGAGCTTCGAGCTTCGTTTG	ATCTTTTGGTGCTCCCACAC
CH02c11[b]	10	B	PET	205–241	TGAAGGCAATCACTCTGTGC	TTCCGAGAATCCTCTTCGAC
CH02d08[b]	11	A	VIC	205–258	TCCAAAATGGCGTACCTCTC	GCAGACACTCACTCACTATCTCTC
CH01f02[b]	12	A	6-FAM	159–214	ACCACATTAGAGCAGTTGAGG	CTGGTTTGTTTTCCTCCAGC
GD147[c]	13	C	PET	127–160	TCCCGCCATTTCTCTGC	GTTTAAACCGCTGCTGCTGAAC
CH04c07[b]	14	B	VIC	95–141	GGCCTTCCATGTCTCAGAAG	CCTCATGCCCTCCACTAACA
CH02c09[b]	15	B	VIC	231–257	TTATGTACCAACTTTGCTAACCTC	AGAAGCAGCAGAGGAGGATG
CH04f10[b]	16	C	6-FAM	173–263	GTAATGGAAATACAGTTTCACAA	TTAAATGCTTGGTGTGTTTTGC
CH01h01[b]	17	B	6-FAM	97–143	GAAAGACTTGCAGTGGGAGC	GGAGTGGGTTTGAGAAGGTT

[a] Silfverberg-Dilworth et al. (2006)

[b] Liebhard et al. (2002)

[c] Hokanson et al. (1998)

A set of 17 microsatellites (Guilford et al. 1997; Hokanson et al. 1998; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) was used (Table 2). Sixteen out of the 17 SSR markers are included in the list proposed by the ECPGR group (Evans et al. 2007), whereas the remaining one (CH01h02) was included as it has been successfully employed before by our team (Santesteban et al. 2009; Urrestarazu et al. 2010, 2011). Each marker belongs to one of the 17 different linkage groups that exist in the apple genome, which ensures independence among loci. The forward primers were labeled with 6-FAM, VIC, NED, or PET fluorescent dye, and three multiplex PCRs, denoted as A, B, and C, were designed (Table 2).

Polymerase chain reactions (PCR) for the three multiplex PCRs were performed in a final volume of 10 μL using 10 ng of DNA template, 0.10 μM of each primer, except for CH02c11 and CH02c06 for which 0.15 and 0.40 μM were used, respectively, and 1× PCR Master mix of QIAGEN kit multiplex PCR (Qiagen, Hilden, Germany). The PCR reactions were carried out in a thermal cycler (model 2720; Applied Biosystems, Foster City, CA, USA) with the following temperature profile for multiplexes A and B: an initial denaturation step at 95°C for 15 min, followed by five touchdown cycles at 95°C for 30 s, 65–1°C/cycle for 1 min and 72°C for 1 min, followed by 30 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 1 min and a final step at 72°C for 30 min. Multiplex C was slightly modified, as touchdown cycle number was raised to seven, and annealing temperature for the last 30 cycles was 58°C instead of 60°C. The PCR products were diluted in 120 μL H_2O and then 1 μL (diluted PCR products) was mixed with 10 μL Hi-di Formamide (Applied Biosystems, Foster City, CA, USA) and 0.5 μL 500-LIZ size standard (Applied Biosystems, Foster City, CA, USA). The fragment analyses were performed on a ABI PRISM 3730 sequencer (Applied Biosystems, Foster City, CA, USA) and PCR products analyzed and sized with Peak Scanner Software ver. 1.0 (Applied Biosystems, Foster City, CA, USA)

Diversity analysis

Genotypes were checked for the presence of null alleles, using Micro-checker ver. 2.2.3 software (van Oosterhout et al. 2004). The number of alleles per locus (A), the number of rare alleles per locus (B, number of alleles with frequency <0.05), the observed (H_o) and expected heterozygosity (Nei's diversity, hereafter H_e) and the number of unique alleles (those present in only one accession) were calculated with the SPAGeDi ver. 1.3 software (Hardy and Vekemans 2002). The effective number of alleles was calculated as $A_e = \left(\sum p_i^2\right)^{-1}$ (Morgante et al. 1994), where p_i represents the frequency of the *i*th allele. The discrimination power was calculated as $PD = 1 - \sum p_i^2$ (Tessier et al. 1999), where p_i represents the frequency of the *i*th genotype. To determine the genetic uniqueness of each accession, and to quantify redundancy, the multilocus DNA profile of all the accessions was compared pairwise. Redundancy was determined as the proportion of distinguishable genotypes (Ellstrand and Roose 1987). We also calculated the percentage of genotypes related by hybridization, considering as such those sharing at least one allele at each locus, and the percentage that could be related by mutation, considering as such those that only differed in one allele, as proposed in Boccacci et al. (2006) and Pereira-Lorenzo et al. (2011).

Analysis of genetic structure

In order to investigate the population structure and to assign individuals to populations based on the SSR genotypes, we used the Bayesian model-based clustering procedure implemented in Structure ver. 2.2.3 (Pritchard et al. 2000), as this software allows performing inference of genetic structure in datasets that include several levels of ploidy. In this study, diploid and triploid material was present and, for the latter, we observed individuals with one, two, or three alleles at each loci. If two alleles are observed at a locus in a triploid individual, either of them may be represented by two allele copies, creating ambiguity. Structure ver. 2.2.3 implements an algorithm that generates appropiate genotypes given both this ambiguity and the one caused by null alleles (Falush et al. 2007). To do so, we used the recessive allele approach (Pritchard et al. 2000), encoding all the individuals as formal triploids: in diploids, the third absent allele was assigned as missing data (−9), whereas in triploids, if needed, one of the alleles detected was duplicated until the declared ploidy was met (Stöck et al. 2010). The tests were done based on an admixture model where the allelic frequencies were correlated and a burn-in period of 200,000 and 500,000 iterations for data collection was applied. The analysis was run for *K* values ranging from two to 10 inferred clusters with 10 independent runs each. In order to assess the best *K* value supported by the data, we used the ΔK method described by Evanno et al. (2005) through Structure harvester ver. 0.6.1 application (Earl 2011) to examine the rate of change in successive posterior probabilities over the range of *K* values. When the results suggested that the *K* groups could be further structured in sub-groups, a second-level (nested) Structure analysis was performed individually for each *K* group (Jacobs et al. 2011; Jing et al. 2010). Genotypes were assigned to the group (or sub-group) for whom they had the highest membership coefficient, considering strong affinity when the assignation probability (*qI*) was ≥0.80 (Breton et al. 2008; Miranda et al. 2010; Pereira-Lorenzo et al. 2010). Modal assignment across all replicate runs was used to determine the final placement of a genotype in a group or

sub-group. The results were displayed graphically in a barplot using Distruct (Rosenberg 2004).

The genetic discrimination in groups and sub-groups found using Structure was confirmed with multivariate analysis (FCA) and analyses of molecular variance (AMOVA). FCA analyses were performed on the diploid material using the "AFC sur populations" module of Genetix 4.05 software (Belkhir et al. 1996–2004) for which rare alleles (frequency <2 %) were removed and considered as missing data, as they may bias analyses (Breton et al. 2008). Finally, to validate the genetic variation between the groups and sub-groups defined by Structure, AMOVA analyses were performed by Genodive (Meirmans and van Tienderen 2004). Descriptive statistics, including variation between groups or sub-groups (F_{ST}) and diversity within sub-groups including H_e, number of polymorphic alleles, and number of private alleles (those present only in one sub-group) were estimated using Genodive (Meirmans and van Tienderen 2004) Additionally, the allelic richness (El Mousadik and Petit 1996) was estimated for the diploid material using FSTAT (Goudet 1995).

Results

SSR polymorphism

All the loci amplified in this study were polymorphic. Due to the absence of amplification product, insufficient fluorescence signal, or unreliable microsatellite profiles when NZ05g08 was used, we decided to remove it from the analyses. Fourteen of the remaining 16 primers (Table 2) had single-locus amplifications, whereas CH01h02 and CH02c11 amplified two loci, as reported in Guarino et al. (2006) and in Garkava-Gustavsson et al. (2008). For both markers, amplification was only considered for the main locus described in Liebhard et al. (2002) discarding the remaining loci. For CH01h02, the secondary locus discarded was much less polymorphic, whereas in CH01c11 the secondary locus was monomorphic. A preliminary analysis with Micro-Checker detected deviations due to null alleles for three loci (Hi02c07, CH04f10, and CH01h01).

The 16 SSR loci amplified a total of 267 alleles (Table 3) in the 538 apple cultivars analyzed (493 local accessions and 45 reference cultivars), varying from 12 (CH01h02 and GD12) to 30 (CH04f10) alleles per locus. The set of local accessions presented all the amplified alleles, whereas in reference cultivars only 56 % (149 alleles) of them were found. The mean number of alleles per locus was 16.69, and the average effective number of alleles was 6.69, ranging from 2.24 (Hi02c07) to 11.04 (CH04f10). We identified a total of 168 rare alleles out of which 32 were present in only one accession (unique alleles), which seems to indicate a substantial level of diversity not previously used for breeding. All accessions, except Negu Sagarra (BMN0013) had any of those rare alleles. Those levels or polymorphism are similar to those reported by van Treuren et al. (2010) for a comparable number of accessions (695) curated at eight Dutch collections.

In total, 73 of the 304 genotypes identified in this study (24 %) were found to be triploids. This percentage was slightly lower than that found for local apple cultivars in Northwestern Spain (29 %) (Ramos-Cabrer et al. 2007). CH02c06 and CH03d07 had the highest number of genotypes with three alleles with 46 and 43 individuals, respectively, whereas CH01h02 and Hi02c07 detected a third allele in only 11 and 13 individuals, respectively. We found 61 genotypes with three alleles in more than one locus, and the remaining genotypes presented only one locus with a third allele. We checked and confirmed that the third allele also existed in other diploid or triploid accessions.

Genetic diversity

H_e summarizes the fundamental genetic variation of a population or species for a single parameter (Berg and Hamrick 1997) and, for this reason, it is a commonly used parameter that allows comparison with the literature. The estimate of genetic diversity in local cultivars of this study (0.82), calculated by gene diversity (H_e), was in agreement with the level of polymorphism reported for local apple cultivars in Northwestern Spain (0.80; Pereira-Lorenzo et al. 2007) and it is slightly higher than that obtained for a reduced set of Spanish apple cultivars from different geographical origins (0.73; Pereira-Lorenzo et al. 2008) or than those reported for Italian, Swedish, and Bosnian material (Garkava-Gustavsson et al. 2008; Gasi et al. 2010; Guarino et al. 2006).

The inbreeding coefficient F_{IS} ranged from 0.399 (CH04f10) to −0.055 (CH01h10) in the overall set, with an average value of 0.043 for all loci (Table 3). Non-significant and negative values were obtained for most loci, which indicates a similar contribution of mutation and migration to genetic variation in the material. The average F_{IS} was also quite low (0.045, $P<0.001$) for the set of local cultivars, suggesting no loss of heterozygosity among the accessions analyzed. It is noteworthy that high F_{IS} values often result from the presence of null alleles (Bruford et al. 1998), which were only detected in three loci. The F_{IS} values (Table 3) were closer to those reported by Pereira-Lorenzo et al. (2007; −0.088) and Gasi et al. (2010; 0.023) in *Malus* x *domestica* germplasm than to those reported for wild *Malus* (Coart et al. 2003; Gharghani et al. 2009; Richards et al. 2009).

Cultivar identification

Comparison of SSR profiles revealed 85 groups of genotypes that had the same SSR profile (marker duplicates). The size of those groups varied from two to 28 genotypes,

Table 3 Measures of genetic diversity at three different levels: overall set of unique accessions, set of local material, and set of reference material

Locus	Overall set (n=304)							Set of local material (n=259)							Set of reference material (n=45)						
	A	B	Ae	Ho	He	P_D	F_{IS}	A	B	Ae	Ho	He	P_D	F_{IS}	A	B	Ae	Ho	He	P_D	F_{IS}
Hi02c07[a]	13	8	2.24	0.51	0.57	0.75	0.136**	13	8	2.21	0.51	0.54	0.72	0.130**	6	1	3.27	0.72	0.70	0.85	0.005
CH02c06	21	13	10.13	0.91	0.90	0.99	0.019	21	13	9.99	0.90	0.90	0.99	0.028	11	4	7.01	0.90	0.86	0.94	−0.033
GD12	12	7	4.04	0.74	0.76	0.91	0.055	12	7	4.09	0.75	0.76	0.91	0.035	6	0	3.58	0.80	0.73	0.88	−0.036
CH05f06	13	5	7.41	0.86	0.87	0.97	0.048*	13	5	7.39	0.88	0.87	0.97	0.024	8	3	3.97	0.85	0.76	0.90	−0.072
CH03d07	22	15	8.37	0.90	0.88	0.98	−0.001	22	16	8.22	0.90	0.88	0.98	0.007	12	6	5.34	0.87	0.83	0.95	0.001
CH04e05	17	13	4.14	0.81	0.76	0.93	−0.044	17	13	4.04	0.80	0.77	0.93	−0.033	9	5	3.11	0.77	0.69	0.87	−0.090
CH01h10	14	10	3.29	0.75	0.70	0.88	−0.055	14	10	3.36	0.76	0.71	0.89	−0.052	6	3	2.62	0.72	0.64	0.77	−0.112
CH01h02	12	8	3.83	0.79	0.74	0.89	−0.023	12	8	3.59	0.77	0.72	0.88	−0.026	7	1	3.83	0.90	0.75	0.85	−0.198
CH02c11	16	8	9.04	0.89	0.89	0.98	0.022	16	8	8.67	0.88	0.88	0.98	0.029	11	4	7.09	0.95	0.86	0.94	−0.113
CH02d08	19	13	6.54	0.84	0.85	0.96	0.034	19	13	6.43	0.84	0.85	0.96	0.030	11	5	6.10	0.82	0.85	0.94	0.093
CH01f02	19	12	8.98	0.88	0.89	0.98	0.032	19	12	8.81	0.88	0.89	0.97	0.027	10	4	6.31	0.85	0.85	0.94	0.060
GD147	13	8	5.41	0.83	0.82	0.95	−0.034	13	8	5.40	0.83	0.81	0.95	−0.023	10	5	4.21	0.87	0.77	0.91	−0.118
CH04c07	17	10	8.01	0.91	0.88	0.97	−0.036	17	11	8.04	0.91	0.87	0.97	−0.034	10	2	7.10	1.00	0.87	0.95	−0.155
CH02c09	13	5	8.28	0.87	0.88	0.97	0.040	13	6	8.34	0.86	0.88	0.97	0.060*	8	2	6.05	0.97	0.84	0.93	−0.161
CH04f10[a]	30	23	11.04	0.59	0.91	0.98	0.399***	30	24	10.70	0.59	0.91	0.97	0.406***	15	8	8.20	0.69	0.88	0.95	0.223**
CH01h01[a]	16	10	6.27	0.82	0.84	0.96	0.065*	16	10	6.31	0.82	0.84	0.96	0.077*	9	4	4.92	0.92	0.80	0.91	−0.136
Mean	16.69	10.50	6.69	0.81	0.82	0.94	0.043***	16.69	10.75	6.60	0.80	0.82	0.94	0.045***	9.31	3.56	5.17	0.85	0.79	0.9	−0.049

Number of alleles per locus (A), number of rare alleles (B), number of effective number of alleles (Ae), observed (Ho), and expected (He) heterozygosity, discriminant power (P_D), and F_{IS} value are included

[a] Locus with null alleles detected with Micro-Checker (van Oosterhout et al. 2004)

*P<0.05, **P<0.01, ***P<0.001

and involved 319 accessions as a whole (Table 4). Therefore, 234 accessions were identified as duplicates and appeared within and among collections. Some duplicates were expected, as the accessions received very similar or identical denominations (e.g. the identity groups with "Pero", "Ortell", and "Verde Doncella"). However, most groups of duplicates comprised accessions that have different names (or whose name is unknown) and also different geographical origin, suggesting that those plants have been spread through grafting. Most of the 234 accessions with a duplicate SSR profile (172) were genetically identical to other local accessions, whereas only 62 (27 %) were genetically identical to 13 of the references. As expected, the reference cultivars for which SSR identities were found were almost exclusively old Spanish cultivars ("Verde Doncella", "Manyaga", "Camuesa", "Ortell", and "Bost Kantoi") or international cultivars introduced in Spain long ago ("Reinette Blanche", "Reine des Reinettes", "Rome Beauty", "Red Delicious", "Stayman", "McIntosh", and "Golden Delicious"). One accession (Isovol-1) had identical marker profile to "Gala", a cultivar introduced in Spain in the 1980s [its identity was phenotypically confirmed (V. Urbina, personal communication)]. The 234 genotypes that were identified as SSR duplicates were not further considered for the analysis of genetic diversity, which was therefore restricted to the remaining 304 (259 local accessions and the 45 reference cultivars).

Within the 304 unique genotypes, small profile differences involving a single allele were found among 16 accessions (Table 5). These minor differences can be due to spontaneous somatic mutations that are known to occur in long-lived trees propagated by grafting (Weissinger 1985). Additionally, 23 pairs of cultivars shared at least one allele per locus (ESM 3), suggesting possible relationships through hybridization. In most cases, those relationships involved only local accessions, but two accessions collected in the province of Lleida (MRFL101 and MRFL103) shared alleles with "Rome Beauty", a cultivar that in the past was quite relevant in that region (Itoiz 2000) and a third accession (MRFL100) could be related to "Golden Delicious". Six of those 23 pairs were composed by a diploid and a triploid accession, and the triploid accession of each pair presented the same genotype of the diploid plus an extra allele in two to 12 loci (ESM 3). The triploid accessions might be the result from the fertilization of an unreduced diploid egg cell (the diploid identified accession) by 1× pollen which carries an allele different to the other two (Einset 1948; Einset 1952; Janick and Moore 1996).

Genetic structure and differentiation

The 304 unique genotypes were analyzed using Structure, and the analysis of the rate of change ΔK over the range of K values (Fig. 1a) showed a clear maximum for K=2 (ΔK= 1,265.6). Higher K values did not show secondary peaks, as ΔK varied from 0.1 to 48.4. This partitioning level corresponds to a very strong differentiation in two major groups, one clustering 159 genotypes (G1) and a second one with 145 (G2). However, the exploration of the results obtained with Structure suggested that the two major groups G1 and G2 could be sub-structured; as for K values higher than 2, the proportion of genotypes assigned to each population was not symmetric, and many accessions were strongly assigned to one population or another, which are both considered strong indications that a real population structure exists (Pritchard et al. 2000). Therefore, each of the two major groups was submitted separately to further Structure analysis (Fig. 2). The analysis of the relationships between K and ΔK for G1 suggested possible K values at K=2 and K=4 (Fig. 1b), and similar results were observed for G2 (Fig. 1c). To assess the robustness of the subdivisions obtained at K=2 and K=4, simulations for each K value were examined for internal consistency between runs and the percentage of accessions that was assigned unambiguously (qI>0.8) to each sub-group was accounted. In G1, the assignment of the genotypes to the sub-groups was well correlated among runs (r>0.95) for both K values, but while for K=2 ca. 80 % accessions showed strong affinity to the sub-groups, less than half had strong affinity for K=4, so K=2 was selected as most suitable. For G2, the assignment of genotypes was well correlated among runs for both K values, but higher overall affinity was found for K=4 (mean $qI_{K=2}$=0.70, mean $qI_{k=4}$= 0.78), thus we adopted K=4 as number of sub-groups for G2. The final six sub-groups considered in this study (G1.1, G1.2, G2.1, G2.2, G2.3, and G2.4) are shown in Fig. 2, together with the location of the reference cultivars at each sub-group. The sub-group each accession was assigned to is indicated in ESM 1. The affinity of most individuals (70 %) to their assigned sub-groups was unambiguous, whereas for the remaining 96 individuals with lower affinities, their placement in a sub-group results from consistent assignments among runs.

The first partition of genetic variation in two strongly differentiated groups (G1 and G2) clustered most old Spanish references (all but "Bost Kantoi") with 153 of the accessions, while the 38 international references (and "Bost Kantoi") were grouped with the remaining 106 accessions (Fig. 2). The examination of the sub-groups shows that those two formed within G1 (G1.1 and G1.2) had similar size, and ≈80 % genotypes at each one had strong affinity to the sub-group. G1.1 included the reference cultivars "Esperiega", "Manyaga", "Verde Doncella", "Ortell", and "Camuesa" (all with strong affinity), while "Geza Miña" was strongly assigned to G1.2. The second major group had two pairs of sub-groups of similar size, and varied proportions of genotypes with strong assignation probability. The smallest groups were G2.1 (23 genotypes), which included "Democrat", "JerseyMac", and "Cox's Orange Pippin", and G2.3, with 22 genotypes distributed evenly among accessions and all the references related with "Golden Delicious" and "Wijcik". The latter sub-group had only three strongly assigned genotypes ("Golden

Table 4 Marker duplicate groups of accessions with the same allelic profile at 16 SSR identified

Marker duplicate group number	Accessions with the same allelic profile at 16 SSR				
1	Totxa (BMZ060)	Ull de Nespre (MRFL097)			
2	Garralda-02 (BMN0157)	Garralda-03 (BMN0158)	Garralda-04 (BMN0159)	Garralda-05 (BMN0160)	
3	**Stayman Winesap**	Solafuente (BMZ074)			
4	**Red Delicious**	Ribera-04 (BMN0286)	Arenas (MRFL010)		
5	Injerta (BMN0303)	Josue (BMN0304)			
6	**McIntosh**	Magistral Roja Lardero (MRFL025)			
7	Reineta Gris (BMN0012)	Manzana Pera (BMN0064)	Beltza (BMN0066)	La de Sidra (BMN0198)	Kapandoja (BMN0269)
8	Lekaroz-01 (BMN0009)	Leitza-03 (BMN0053)	Leitza-05 (BMN0055)	Libra Sagarra (BMN0258)	Urarte (BMZ063)
9	Olazti-01 (BMN0101)	Aranache-09 (BMN0218)			
10	Manda Burua (BMN0033)	Leitza-02 (BMN0052)	La de la Grasa (BMN0104)	Manzana Color Rosa (BMN0111)	Iturmendi-08 (BMN0123)
	Osto- Zabala (BMN0126)	La Amarilla (BMN0145)	Mendioroz-01 (BMN0206)	Aranache-19 (BMN0229)	Palestiña (BMN0261)
	Prau-Riu 4 (BMZ071)	Iregua Alberite (MRFL011)	San Juan (MRFL022)	Les-2 (MRFL074)	Bossost-5 (MRFL079)
11	**Reinette Blanche**	Azpirotz-02 (BMN0065)	Reina Reineta (BMN0103)	Arakil-03 (BMN0144)	Pampandoja (BMN0195)
	Ibero-04 (BMN0197)	Campanas-01 (BMN0199)	Aranache-05 (BMN0214)	Aranache-07 (BMN0216)	Aranache-08 (BMN0217)
	Ayegui-01 (BMN0242)	Eulz-01 (BMN0244)	Sansol-01 (BMN0248)	Ribera-08 (BMN0290)	Villanuño (BMN0305)
	Constancio (BMN0306)	Urra-01 (BMN0310)	Urra-04 (BMN0313)	Urra-05 (BMN0314)	Reineta Blanca del Canada (BMZ044)
	Reineta Blanca del Canada (BMZ045)	Reineta Blanca del Canada (BMZ046)	Reineta Inesita de Asua (BMZ049)	Navalmoral de Bejar-3 (MRFL030)	Cabdellá-1 (MRFL048)
	Vinçada Tardía (MRFL061)	Gabacha (MRFL071)	Isovol-2 (MRFL089)		
12	Apez Sagarra (BMN0016)	Aria-03 (BMN0151)	Samuesa (BMN0192)	Eparoz-01 (BMN0202)	Muneta-01 (BMN0245)
	Puente La Reina-03 (BMN0252)	Reineta del Camino (BMN0307)	Urra-02 (BMN0311)	Urra-03 (BMN0312)	Urra-07 (BMN0316)
	Urra-08 (BMN0317)				
13	**Reine des Reinettes**	Urdiain-01 (BMN0114)	Genaro Sagarra (BMN0128)	Valsaina (BMZ075)	
14	Errotz-03 (BMN0138)	Orbaitzeta-02 (BMN0148)	Cabredo-01 (BMN0208)	Aranache-04 (BMN0213)	Izurzu-01 (BMN0236)
	Audiena de Oroz (BMZ005)				
15	Larrenz (BMN0156)	Pero de Sanguesa (BMN0275)	Peruco de Caparroso (BMZ041)		
16	**Bost Kantoi**	Bost Kantoia (BMZ009)			
17	Atxon Sagarra (BMN0040)	Mugiro-04 (BMN0077)	Manzana de Ramas Largas (BMN0107)	Goxoa (BMN0127)	
18	Farrera (BMZ023)	Bossost-2 (MRFL076)			
19	Gigant Lard-1 (MRFL001)	Gigant Lard-2 (MRFL007)			
20	Peangon-Nonsur (BMN0201)	Transparente (BMN0281)	Navarra-05 (BMN9004)		
21	Colorada (BMN0088)	Aranache-17 (BMN0227)			
22	San Bartolomé Sagarra (BMN0008)	Eparoz-02 (BMN0203)	Eparoz-03 (BMN0204)	Inesita de Asua (BMN0268)	Marquinez (BMZ031)
	Reineta Gris del Canada (BMZ048)	Vimianzo-1 (MRFL026)	Bossost-1 (MRFL075)		
23	Asiain-01 (BMN0191)	Reineta Encarnada (BMN0266)	Reineta Encarnada (BMZ073)	Acedas Roja (MRFL003)	

Table 4 (continued)

Marker duplicate group number	Accessions with the same allelic profile at 16 SSR				
24	Viloria-02 (BMN0239)	Puente La Reina-01 (BMN0250)			
25	Landetxo (BMN0270)	Astrakan roja (BMZ004)	Landetxo (BMZ028)		
26	Leitza-09 (BMN0059)	Gurea Arriak (BMN0067)	Urdiain-02 (BMN0115)		
27	Gollano-04 (BMN0235)	Uterga-01 (BMN0253)	Hierro (BMN0267)	Hierro (BMZ027)	
28	Uztegi-02 (BMN0061)	Manzana Pera (BMN0084)			
29	Mugiro-03 (BMN0076)	Mugiro-05 (BMN0078)	Aranache-14 (BMN0223)	Aranache-18 (BMN0228)	Reineta de Regil (BMN0262)
	Reineta Regil (BMZ047)				
30	Eugenia (BMZ022)	Eugenia (MRFL095)			
31	Kampanduxi (BMN0068)	Ochagavia-01 (BMN0181)			
32	Erreka Sagarra (BMN0022)	Motela (BMN0062)			
33	Urte Bete (BMN0265)	Pero de Cervera (BMN0274)	Pero Pardo (BMN0276)	Peruco de Caparroso (BMN0277)	Pera de Sangüesa (BMZ038)
	Pero Pardo (BMZ039)	Moceta (MRFL013)			
34	Azpirotz-01 (BMN0063)	Azpirotz-03 (BMN0069)			
35	Beasoain-02 (BMN0185)	Ayegui-02 (BMN0243)			
36	Kerienlutxia (BMN0042)	Blanca (BMN0086)	Baldano (BMN0124)		
37	Fuerte (BMN0167)	Manda Burua (BMN0169)			
38	Arizkun-01 (BMN0018)	Naranjina (BMN0087)	Colorada (BMN0164)		
39	Errotz-04 (BMN0139)	La de Agua (BMN0189)	Viloria-01 (BMN0238)	Peruco (BMN0246)	Normanda (BMZ035)
40	Zuazu-01 (BMN0207)	Pero (BMN0273)			
41	Leitza-06 (BMN0056)	Leitza-07 (BMN0057)			
42	Anís Sagarra (BMN0014)	Ziordia-02 (BMN0095)	Ziordia-04 (BMN0098)		
43	Ezti-Beltza (BMN0004)	Kamutx Gorrilla (BMN0024)	Aizpurua Sagar (BMN0029)	Patzolua (BMN0260)	
44	Martell (BMN0105)	Agosteña Alberite (MRFL005)			
45	Sant Jaume (BMZ056)	Santa Margarida (BMZ058)			
46	Gar Zabala (BMN0007)	Negra (BMN0080)	Beltza (BMN0085)		
47	Gaya (MRFL020)	Gotarta-1 (MRFL064)			
48	Negu Sagarra (BMN0013)	Urte-Bete (BMN0031)	Usta Beltza (BMN0038)	Sagar Sagarra (BMN0039)	Esporotarra (BMN0046)
	Olivo Sagarra (BMN0081)	Iturmendi-01 (BMN0116)			
49	Zuria (BMN0132)	Aranache-02 (BMN0211)	Aranache-12 (BMN0221)		
50	Urte Sagarra (BMN0017)	Geza Gorritxa (BMN0034)	Txori Sagarra (BMN0129)	Urtebete (BMZ064)	
51	Navalmoral de Bejar-1 (MRFL028)	Navalmoral de Bejar-2 (MRFL029)			
52	Aranache-03 (BMN0212)	Gollano-02 (BMN0232)			
53	Ribera-01 (BMN0283)	Ribera-02 (BMN0284)	Ribera-03 (BMN0285)	Lahoz-01 (BMN0299)	
54	Aranache-01 (BMN0210)	De pera (BMZ018)	Pera-2 (BMZ037)		
55	Maestro Sagarra (BMN0131)	Aranache-16 (BMN0226)	Gollano-03 (BMN0234)	Puente La Reina-02 (BMN0251)	
56	La Roja (BMN0133)	Aranache-21 (BMN0231)			
57	**Camuesa**	Izurdiaga-01 (BMN0135)	La del Picorcillo (BMN0143)	Beasoain-01 (BMN0184)	Ibero-01 (BMN0193)

Table 4 (continued)

Marker duplicate group number	Accessions with the same allelic profile at 16 SSR				
	Viella-1 (MRFL080)				
58	De Agosto (MRFL057)	Camosa (MRFL060)			
59	Ciri blanc (BMZ017)	Del Ciri (BMZ019)	Cul de Cirio (MRFL039)		
60	**Manyaga**	Cirio (MRFL033)	Magaña (MRFL043)	Almenar-1 (MRFL045)	Reguard-1 (MRFL053)
	Morro de Conill (MRFL058)	Irgo-2 (MRFL066)	Manyaga (MRFL081)		
61	Calviña Agostera (BMN0102)	Errotz-02 (BMN0137)	Roja (BMN0175)		
62	Cabello de angel (BMZ010)	Cabello de angel (BMZ011)			
63	Uharte Arakil-01 (BMN0134)	Signatillis (BMZ057)			
64	Botil Sagarra (BMN0045)	Botille (BMN0079)	Blanca (BMN0091)	Gordoncha (BMN0092)	
65	Pero Mingan (BMN0278)	Peruco (MRFL002)			
66	Miguela de Ademuz (BMN0271)	Morro de Liebre (BMN0272)			
67	**Ortell**	Ibero-02 (BMN0194)	Ortell (BMN0279)	Ortell (MRFL004)	Ortell (MRFL024)
	Ortell (MRFL037)				
68	Txori Sagarra (BMN0026)	Orache (BMN0280)	Helada (BMZ026)		
69	**Verde Doncella**	Artazcoz-02 (BMN0190)	Sansol-02 (BMN0249)	Verde Doncella (MRFL036)	De Gel (MRFL041)
70	Ama Birgen Sagarra (BMN0032)	Sagar Gorria (BMN0051)	Satrustegui-01 (BMN0146)	Sandia (BMZ055)	
71	Viloria-03 (BMN0240)	Reneta (BMZ050)	Roja del Valle de Benejama (BMZ051)	Zamora-2 (MRFL008)	Zamora-1 (MRFL009)
72	San Miguela (BMN0089)	Ascara-1 (BMZ002)			
73	Santiagina (BMN0125)	Transparente blanca (BMZ062)	Bellaguardia (MRFL027)		
74	Ziordia-01 (BMN0094)	Iturmendi-05 (BMN0120)	Iturmendi-06 (BMN0121)	Iturmendi-07 (BMN0122)	Arakil-02 (BMN0142)
	Galar-01 (BMN0182)	Aranache-11 (BMN0220)	Transparente (BMZ061)	Isovol-3 (MRFL090)	
75	**Rome Beauty**	Reineta del Pozo (BMN0308)	Urra-06 (BMN0315)	Reguard-4 (MRFL056)	Taüll-2 (MRFL068)
76	Belllvei-1 (MRFL059)	Belllvei-2 (MRFL062)			
77	Navarra-01 (BMN9000)	Navarra-04 (BMN9003)	Navarra-06 (BMN9005)		
78	Garisoain-01 (BMN0233)	Manzana Enana (BMN0241)			
79	Baztandarra (BMN0165)	Baztan Sagarra (BMN0173)			
80	Cua Llarga (BMZ024)	Cua llarga (MRFL094)			
81	Morro de Liebre (BMZ032)	Morro de Liebre (BMZ033)			
82	Sagar Txuria (BMN0041)	Iturmendi-02 (BMN0117)			
83	Balaguer Flix-1 (MRFL034)	Torms-1 (MRFL104)			
84	**Gala**	Isovol-1 (MRFL087)			
85	**Golden Delicious**	Durró-1 (MRFL072)			

The reference material was indicated in bold

Table 5 Groups of genotypes that only differed at one allele. Bold and italics highlight the alleles that were different, and underline indicates reference cultivar

Group	Code	Genotype	Ploidy	Hi02c07			CH02c06			GD12			CH05f06			CH03d07			CH04e05			CH01h10		CH01h02	
1	14	Gloster	2n	113	115		236	250		148	154		173	183		205	225		177	206		100	100	234	244
1	BMN0163	De Mine	2n	113	115		236	250		148	154		173	183		205	225		177	206		100	100	234	244
2	19	Reinette Blanche	3n	113	115		230	250	252	150	152		175	179	183	205	213	225	177	204		93	100	234	246
2	BMN0170	Pampandoja	3n	113	115		230	250	252	150	152		175	179	183	205	213	225	177	204		93	100	234	246
3	BMN0192	Samuesa	3n	103	115	148	***244***	252	260	150	150	150	175	183		185	205	225	177	204		93	100	234	246
3	BMZ067	Carapanón	3n	103	115	148	***242***	252	260	150	150	150	175	183		185	205	225	177	204		93	100	234	246
4	BMN0080	Negra	3n	113	113	113	242	250		148	150		165	181	187	201	205	207	177	200	217	100	105	234	242
4	BMN0093	Ostiz-01	3n	113	113	113	242	250		148	150		165	181	187	201	205	207	177	200	217	100	105	234	242
5	BMZ052	San Felipe	2n	113	113		236	258		150	154		181	181		191	223		177	200		93	100	234	238
5	MRFL005	Agosteña Alberite	2n	113	113		236	258		150	154		181	181		191	223		177	200		93	100	234	238
6	BMN0060	Uztegi-01	2n	113	113		230	246		150	150		173	179		191	225		177	177		93	100	242	246
6	BMN0283	Ribera-01	2n	113	113		230	246		150	150		173	179		191	225		177	177		93	100	242	246
7	BMN0112	Manzana Melocotón	2n	107	148		208	252		152	154		173	175		205	217		204	225		100	135	234	234
7	BMZ061	Transparente	2n	107	148		208	252		152	154		173	175		205	217		204	225		100	135	234	234
8	BMN0041	Sagar Txuria	2n	148	150		208	***244***		161	183		171	181		185	205		225	227		104	117	234	234
8	BMN054	Leitza-04	2n	148	150		208	***242***		161	183		171	181		185	205		225	227		104	117	234	234

Group	CH02c11			CH02d08			CH01f02			GD147			CH04c07			CH02c09			CH04f10			CH01h01		
1	209	217		213	217		179	191		142	142		121	134		241	***251***		193	241		115	117	
1	209	217		213	217		179	191		142	142		121	134		241	***253***		193	241		115	117	
2	209	217	231	211	229	254	179	181		142	152		107	111	113	231	243	253	243	243	243	***101***	115	130
2	209	217	231	211	229	254	179	181		142	152		107	111	113	231	243	253	243	243	243	***111***	115	130
3	209	217	219	205	254		183	183	183	142	142	142	107	113		241	243	255	193	241	243	111	130	
3	209	217	219	205	254		183	183	183	142	142	142	107	113		241	243	255	193	241	243	111	130	
4	217	233		211	225	254	173	183	187	144	158		97	101	121	231	***249***	251	201	205	207	111	117	119
4	217	233		211	225	254	173	183	187	144	158		97	101	121	231	***247***	251	201	205	207	111	117	119
5	217	225		213	248		183	207		142	144		95	121		***247***	251		241	241		117	119	
5	217	225		213	248		183	207		142	144		95	121		***249***	251		241	241		117	119	
6	231	239		225	254		179	187		135	142		97	113		231	253		191	***225***		119	130	
6	231	239		225	254		179	187		135	142		97	113		231	253		191	***191***		119	130	
7	217	231		254	254		181	201		142	152		109	113		237	***251***		239	243		113	130	
7	217	231		254	254		181	201		142	152		109	113		237	***255***		239	243		113	130	
8	217	227		217	246		179	187		152	154		95	111		243	253		241	241		113	113	
8	217	227		217	246		179	187		152	154		95	111		243	253		241	241		113	113	

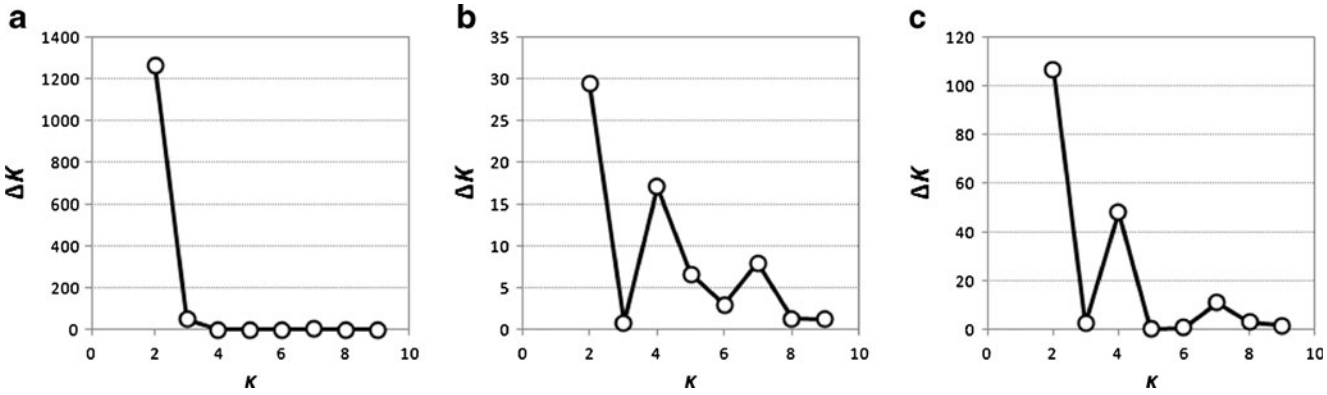

Fig. 1 Exploration of K value for Structure analysis of apple germplasm by estimates of the rate of change of the slope of the log likehood curve (ΔK) calculated according to Evanno et al. (2005) plotted against K. **a** Plot for the analysis on the 304 unique genotypes giving a robust ΔK maximum at K=2 and two groups (G1 and G2). **b** Plot for the sub-structure analysis for G1 and **c** plot for the sub-structure analysis of G2

Delicious", "Charden", and Montcada-3) and the rest of the genotypes assigned to this sub-group had qI>0.65. G2.4 was the sub-group that included a highest proportion of accessions (92 %), and also the highest proportion of strongly assigned genotypes (70 %). Three references, namely the old international cultivars "Red Boskoop", "Gravenstein", and "McIntosh", were included here. Finally, the sub-group G2.2 included the 20 of the remaining 21 references and 29 accessions. Three genotypes clustered in G2 (Bossost-4 and Santiaguera and the scab-resistant reference cultivar "Prima") could not be consistently assigned to any sub-group, as their qI was ≈0.3 for G2.1, G2.3, and G2.4. "Prima" is a cultivar obtained by multiple crosses including *Malus floribunda* 821, "Golden Delicious", and "Rome Beauty", among others (Dayton et al. 1970), and this multiple origin could explain it admixes to different sub-groups.

Based on AMOVA analysis, significant variance differences were also found among the six sub-groups previously identified by the nested-Bayesian clustering (Table 6). Genetic differentiation among sub-groups accounted for 11.1 % of the variation (14.6 % when AMOVA was performed for genotypes with strong affinity to a sub-group). The overall F_{ST} value of 0.111 suggested a moderate but highly significant (P<0.001) differentiation between sub-groups. This value was higher than that obtained when the differentiation between major groups (G1 and G2) was considered (F_{ST}=0.076, P<0.001). The genetic differentiation between the four sub-groups defined by the nested

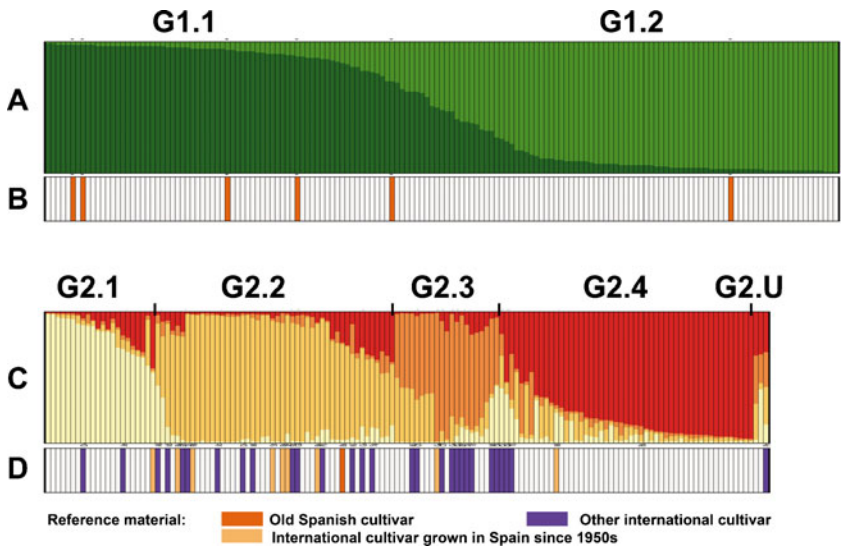

Fig. 2 Substructuring of K=2 Structure groups and placement of reference cultivars. **a** Nested Structure analysis for the first group (G1) identified by the average of 10 independent simulations with K value of 2. **b** and **d** Placement of the reference cultivars according to the key below. **c** Nested Structure analysis for the second group (G2) identified by the average of 10 independent simulations with K value of 4. Genotypes in G2 that could not be consistently assigned to any sub-group are labeled as G2.U. Genotypes have been placed in order according to their average representation within the sub-group

Structure analysis for G2 was much higher (F_{ST}=0.115, P<0.001) than that obtained between the two sub-groups in G1 (F_{ST}=0.045, P<0.001). Genetic discrimination between sub-groups was also confirmed through FCA analysis, as the six sub-groups could be easily differentiated along the three axes of the FCA graphic (Fig. 3). The sub-groups formed exclusively by local and old Spanish material were placed in the negative zone of the first axis, whereas the rest were placed in the positive region.

Genetic diversity indexes were calculated for the Structure sub-groups (Table 7). H_e varied from 0.72 to 0.81, revealing a high proportion of heterozygous individuals in the six sub-groups. The proportion of alleles represented in each sub-group varied from 44 % (G2.3) to 81 % (G1.1). All sub-groups had at least two private alleles (i.e. present in only one sub-group), but they were mostly concentrated in G1.1 and G2.4. Moreover, most of the private alleles were also unique, as they were present in only one accession. Since the larger genetic diversity found at the larger sub-groups could be due partially to their size, we calculated their allelic richness (El Mousadik and Petit 1996) to properly compare the allelic diversity of the sub-groups, as this rarefaction method performs scaling down to the smallest group (N=22 in this study) to compensate differences in size. The higher allelic richness values observed for sub-groups G1.1 and G2.4, and the smaller one of G2.3 confirmed their diversity (Table 7).

In order to test the correspondence between the geographic origin of the accessions and their placement in the six sub-groups revealed in this study, the accessions, collected throughout 11 autonomous communities (the first level of administrative division in Spain), were grouped into three greater regions defined by proximity and traditional agricultural relations between them: Northwestern Spain (NW), and two regions in Northeastern Spain (NE-1 and NE-2). NW region included the few unique accessions that had been collected in Galicia, Asturias, Cantabria, and Castilla y Leon, NE-1 those collected in Navarre, Basque Country, and La Rioja, and, NE-2 region included those from Aragon, Catalonia, Valencia, and Balearic Islands.

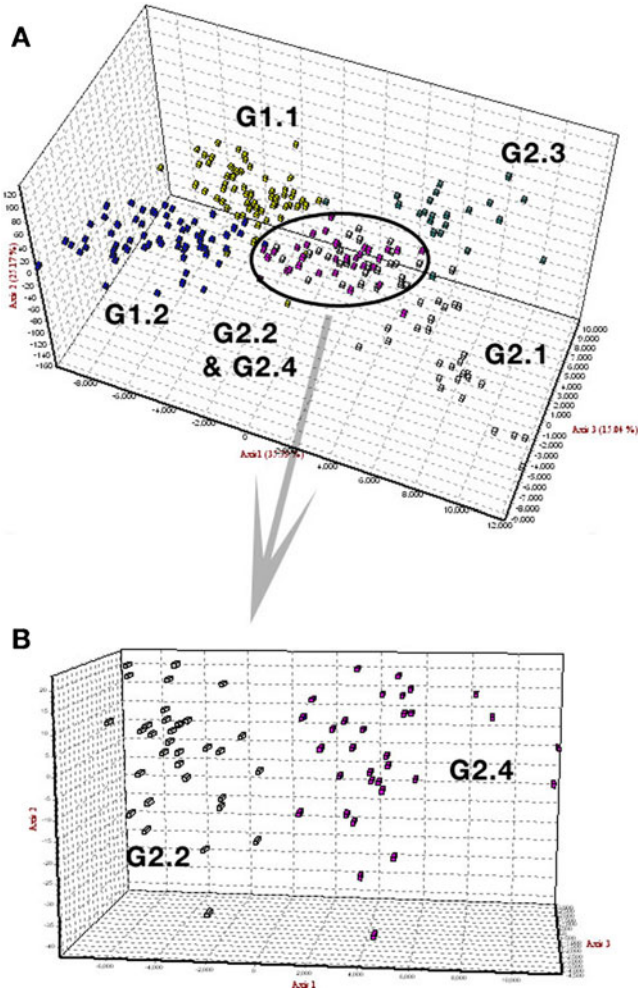

Fig. 3 **a** Factorial correspondence analysis (FCA) based on polymorphism at 16 SSR loci for 231 diploid genotypes (194 accessions and 37 reference cultivars). Accession color reflects the consistent assignment using Bayesian analysis to the sub-groups defined in Fig 2. **b** Close-up of the FCA for the diploid genotypes included in Bayesian sub-groups G2.2 and G2.4 defined in Fig. 2

Table 6 Analysis of molecular variance (AMOVA) based on the 16 SSR loci of 304 Spanish apple accessions and reference cultivars corresponding to the groups and sub-groups defined by Structure analysis

Populations	All genotypes					Genotypes with qI >0.8				
	df[a]		Variance components (%)			df		Variance components (%)		
	W[b]	A[c]	W	A	p value	W	A	W	A	p value
2, Acessions and references	225	1	94.6	6.0	0.001					
2, Major groups G1 and G2	225	1	92.4	7.6	0.001	159	1	88.4	11.6	0.001
2, Sub-groups of G1	118	1	95.7	4.3	0.001	93	1	93.7	6.3	0.001
2, Sub-groups of G2	105	1	92.3	7.3	0.001	59	1	83.3	16.7	0.001
4, Sub-groups of G1	116	3	92.6	7.4	0.001	53	3	86.7	13.3	0.001
4, Sub-groups of G2	103	3	88.5	11.5	0.001	47	3	83.0	17.0	0.001
6, Sub-groups of G1 and G2	221	5	88.9	11.1	0.001	119	5	85.4	14.6	0.001

[a] df degrees of freedom
[b] W within populations
[c] A among populations

Although there was not a straightforward correspondence between the geographic origin of the accessions and their clustering, some trends can be pointed out (Table 8). The accessions collected in NW region clustered mostly in G1, evenly distributed between its two sub-groups. The NE-1 and NE-2 regions were represented in all sub-groups, but in different proportions: the accessions from the NE-2 region were particularly frequent on sub-groups G1.1 and G2.3, whereas the accessions from the NE-1 region were the commonest (>75 %) in sub-groups G1.2, G2.1, and G2.4.

Discussion

SSR characterization

Reliable genetic markers are essential for efficient differentiation of cultivars and to establish the genetic relationships among them. All SSR loci analyzed in this study displayed a high degree of polymorphism with 12–30 alleles per locus. The overall allelic diversity showed by the set of 16 SSR used reveals a high genetic variation in the apple germplasm evaluated. When compared to other wide scale studies of apple genetic diversity, our average number of alleles per locus (16.69) was similar to that reported (18.50) by van Treuren et al. (2010) in their analysis of 695 local genotypes from several Dutch collections, and higher than the one obtained (12.30) by Pereira-Lorenzo et al. (2007) and Gharghani et al. (2009) who studied both pools of ≈100 local and commercial genotypes. Those levels or polymorphism reveal a high genetic diversity in the studied apple germplasm, in agreement to the level of polymorphism reported by van Treuren et al. (2010) and by Pereira-Lorenzo et al. (2007). Besides, diversity levels were slightly higher than those reported for Italian, Swedish, and Bosnian material (Garkava-Gustavsson et al. 2008; Gasi et al. 2010; Guarino et al. 2006).

Collection management

Based on the level of variation observed in the study, the estimated probability of observing identical multilocus SSR profiles by chance was 1.61×10^{-17}, indicating a high resolution potential of the marker set we used. Consequently, those accessions with identical SSR profiles may be considered duplicates in terms of collection management. As a whole, the potential redundancy found among the accessions curated in the three collections (Pamplona, Zaragoza, and Lleida) was around 47 % due, in a similar proportion, to duplications within and among collections. This value is similar to that reported for other apple or pear collections (Bassil et al. 2008; Pereira-Lorenzo et al. 2008; Ferreira dos Santos et al. 2011), and much higher than the 32 % of redundancy reported for eight apple collections in the Netherlands (van Treuren et al. 2010) which, nevertheless, could have been underestimated as only one of their collections was fully analyzed. Despite the overlap between our collections, they were ≈50 % unique.

High levels of duplication within a collection must be avoided due to the high maintenance cost of field collections (Hokanson et al. 1998), but a certain level of duplication provides a safety backup system. The SSR profiles obtained in this study, along with morpho-agronomic evaluation (in progress) will be used to critically re-examine the composition of the collections and the possibility of removal of certain accessions duplicated within collections. The results we obtained constitute a good example on how cooperation mechanisms among collections with coordinated actions can improve collection management efficiency. Moreover, in our case, the cooperation among Spanish apple collections has

Table 7 Descriptive information for each of the six sub-groups of genotypes identified by Structure analyses

	Number of genotypes in the sub-group			He	Number of alleles					Allelic richness[d]
Sub-group	Accessions	References[a]	Genotypes with $qI>0.8$		Total	Private[b]	Unique[c]	A	A_D	
1.1	73	5	63	0.80	215	19	12	13.44	12.80	9.82
1.2	80	1	65	0.76	168	3	2	10.50	9.40	7.62
2.1	20	3	14	0.80	132	3	1	8.25	8.25	8.03
2.2	29	20	29	0.76	144	3	2	9.00	8.06	7.33
2.3	10	12	3	0.72	117	2	3	7.31	7.00	7.00
2.4	45	3	34	0.81	196	16	12	12.25	10.25	9.28

Summary statistics include the number of individuals in each sub-group, expected heterozygosity (He), total, private, unique, average number of alleles (A) and average number of alleles in diploid genotypes (A_D). Allelic richness is scaled to the smallest group (G2.3 N=22)

[a] See Table 2 for details

[b] Alleles detected only in that sub-group

[c] Alleles detected only in one accession

[d] Calculated for diploid genotypes only

been recently increased through an R&D coordinated project that currently involves seven apple collections (including the three used in this study) integrated in the Network of Collections of the Spanish National Program of Plant Genetic Resources. One of the objectives of this cooperation is the joint determination of a core subset representative of the diversity conserved at all those collections. A duplicate of this core subset will be planted at each collection in order to minimize the risk of losing diversity and, in a second stage, it will serve to assess properly the stability of the phenotypes under different sites.

Genetic structure and differentiation

Bayesian clustering analyses have proven to be powerful tools to analyze the genetic structure in tree species such as pear (Ferreira dos Santos et al. 2011; Iketani et al. 2010; Miranda et al. 2010; Volk et al. 2006), plum (Horvath et al. 2011), sweet cherry (Mariette et al. 2010), olive (Breton et al. 2008; Erre et al. 2010) or chestnut (Pereira-Lorenzo et al. 2010). In apple, it had been successfully used to reveal genetic structure mainly in wild *Malus* species such as *M. sieversii* (Richards et al. 2009), *M. orientalis* (Volk et al. 2009) and *M. sylvestris* (Coart et al. 2003; Larsen et al. 2006).

Our study has revealed the existence of a clear structure, not only among the cultivars (Spanish and international) used as reference, but also within the local accessions curated in the three collections in Pamplona, Zaragoza, and Lleida. To explore the genetic structure of cultivated apple in Northeastern Spain, we used a hierarchical approach, starting with a low, but very robust value (K=2), which reflects major division in apple germplasm. The clustering of an important part of local accessions (around 60 %) by Structure analysis in a separate gene pool (G1) independent from the one containing international reference cultivars (G2) may suggest the uniqueness of this local material. The success of nested Bayesian study varied depending upon the group analyzed, and its power became clear when the sub-structuring of G1 was explored, a deep division among local material within two robust sub-groups being revealed. Though there was not a straightforward correspondence between the geographic origin of the accessions and their sub-group placement, which agrees to the traditional exchanges of plant material through grafting in this area (mainly at the Ebro Valley provinces), certain trends could be observed, as the accessions from Aragon and Catalonia were particularly frequent on sub-groups G1.1 and G2.3, whereas G1.2, G2.1, and G2.4 were mainly (>75 %) composed by accessions from Navarre and Basque Country (Table 8). The sub-structure of G2 showed a considerably larger differentiation among sub-groups than the observed for G1 (Table 6) and it is coherent with the presence in this group of the heterogeneous and very diverse group of reference international cultivars. The presence of local accessions in G2 sub-groups could be partly explained by possible incursions of foreign material into local germplasm, resulting in new varieties by hybridization that could have produced lineages that were subsequently propagated through clones (Pereira-Lorenzo et al. 2010, 2011). In our study, two accessions (MRFL101 and MRFL103) could be derived from “Rome Beauty”, a cultivar that in the past was quite relevant (Itoiz 2000) whereas MFRL100 could be derived from “Golden Delicious”, a relatively widespread cultivar in Spain since the 1950s. Several examples of introgression had been previously detected in local Spanish germplasm (Ramos-Cabrer et al. 2007).

Table 8 Geographic origin of the unique local accessions clustered within each sub-group determined by Structure analysis

Sub-group	Region		
	NW[a]	NE-1[b]	NE-2[c]
1.1	7	28	38
1.2	6	69	6
2.1	2	15	3
2.2	–	19	10
2.3	–	3	7
2.4	2	34	8

Geographic regions were defined pooling (by proximity and traditional agricultural relations between them) the Autonomous Communities (AC, the first level of administrative division in Spain) in which accessions were collected

[a] Accessions collected in the AC of Galicia, Asturias, Cantabria, and Castilla y Leon

[b] Accessions collected in the AC of Navarre, Basque Country, and La Rioja

[c] Accessions collected in the AC Aragon, Catalonia, Valencia, and Balearic Islands

The results obtained through the FCA and AMOVA analyses, were coherent with those obtained by Structure revealing moderate, but significant, differentiation among the six sub-groups. The identification of private alleles and remarkable differences in allelic richness among sub-groups constitute further evidence of the genetic structure and of the presence of unique material.

This large-scale comparison of local material from Northeastern Spain with a high number of reference cultivars, chosen to represent a wide range of genetic diversity, can help us to better understand the genetic variation of traditional cultivars from this area and to make a finer delineation of this unique gene pool. Our results highlight the relevance of autochthonous apple germplasm as a reservoir of genetic diversity, the local material analyzed being a good example of genetic distinctness with respect to the main apple cultivars used in European orchards.

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References

Bassil N, Postdam K, Dolan S, Lawliss L (2008) Molecular fingerprints identify historic pear trees in two U.S. National Parks. Acta Hortic 800:417–422

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (1996–2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, Université de Montpellier II, Montpellier, France

Berg EE, Hamrick JL (1997) Quantification of genetic diversity at allozyme loci. Can J For Res 27:415–424

Boccacci P, Akkak A, Botta R (2006) DNA typing and genetic relations among European hazelnut (*Corylus avellana* L.) cultivars using microsatellite markers. Genome 49:598–611

Breton C, Pinatel C, Médail F, Bonhomme F, Bervillé A (2008) Comparison between classical and Bayesian methods to investigate the history of olive cultivars using SSR-polymorphisms. Plant Sci 175:524–532

Brooks RM, Olmo HP (1991) Register of new fruit and nut varieties list 35. HortScience 26:951–978

Brooks RM, Olmo HP (1994) Register of new fruit and nut varieties list 36. HortScience 29:942–969

Bruford MW, Ciofi C, Funk SM (1998) Characteristics of microsatellites. In: Karp A, Isaac PG, Ingram SI (eds) Molecular tools for screening biodiversity. Chapman and Hall, London, pp 202–205

Coart E, Vekemans X, Smulders MJM, Wagner I, van Huylenbroeck J, van Bockstaele E, Roldan-Ruiz I (2003) Genetic variation in the endangered wild apple (*Malus sylvestris* (L.) Mill.) in Belgium as revealed by amplified fragment length polymorphism and microsatellite markers. Mol Ecol 12:845–857

Dayton DF, Mowry JB, Hough LF, Bailey CH, Williams EB, Janick J, Emerson FH (1970) Prima an early fall red apple with resistance to apple scab. Fruit Var Hort Dig 24:20–22

Earl DA (2011) Structure harvester v0.6.1. Available at http://taylor0.biology.ucla.edu/structureHarvester/

Einset J (1948) The ocurrence of spontaneous triploids and tetraploids in apples. Proc Am Soc Hortic Sci 51:61–63

Einset J (1952) Spontaneous polyploidy in cultivated apples. Proc Am Soc Hortic Sci 59:291–302

El Mousadik A, Petit RH (1996) High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic of Morocco. Theor Appl Genet 92:832–839

Ellstrand NC, Roose ML (1987) Patterns of genotypic diversity in clonal plants species. Am J Bot 74:123–131

Erre P, Chessa I, Muñoz-Diez C, Belaj A, Rallo L, Trujillo I (2010) Genetic diversity and relationships between wild and cultivated olives (*Olea europaea* L.) in Sardinia as assessed by SSR markers. Genet Resour Crop Evol 57:41–54

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Evans KM, Fernández F, Laurens F, Feugey L, van de Weg WE (2007) Harmonising fingerprinting protocols to allow camparisons between germplasm collections. Eucarpia XII Fruit Section Symposium, Zaragoza (Spain), pp 16–20

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Ferreira dos Santos AR, Ramos-Cabrer AM, Díaz-Hernández MB, Pereira-Lorenzo S (2011) Genetic variability and diversification process in local pear cultivars from Northwestern Spain using microsatellites. Tree Genet Genomes 7:1041–1056

Food and Agriculture Organization of the United Nations (2011) FAO statistics database on the World Wide Web http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Accessed 25 September 2011

Food and Agriculture Organization of the United Nations/International Plant Genetic Resources Institute (2001) Multi-crop passport descriptors. FAO/IPGRI, Rome, Italy

Garkava-Gustavsson L, Brantestam AK, Sehic J, Nybom H (2008) Molecular characterisation of indigenous Swedish apple cultivars based on SSR and S-allele analysis. Hereditas 145:99–112

Gasi F, Simon S, Pojskic N, Kurtovic M, Pejic I (2010) Genetic assessment of apple germplasm in Bosnia and Herzegovina using microsatellite and morphologic markers. Sci Hortic 126:164–171

Gharghani A, Zamani Z, Talaie A, Oraguzie NC, Fatahi R, Hajnajari H, Wiedow C, Gardiner SE (2009) Genetic identity and relationships of Iranian apple (*Malus* x *domestica* Borkh.) cultivars and landraces, wild *Malus* species and representative old apple cultivars based on simple sequence repeat (SSR) marker analysis. Genet Resour Crop Evol 56:829–842

Gianfranceschi L, Seglias N, Tarchini R, Komjanc M, Gessler C (1998) Simple sequence repeats for the genetic analysis of apple. Theor Appl Genet 96:1069–1076

Goudet J (1995) FSTAT, a program for INM PC compatibles to calculate Weir and Cockerham's (1984) estimators of F-statistics. J Hered 86:485–486

Guarino C, Santoro S, De Simone L, Lain O, Cipriani G, Testolin R (2006) Genetic diversity in a collection of ancient cultivars of apple (*Malus* x *domestica* Borkh.) as revealed by SSR-based fingerprinting. J Hortic Sci Biotechnol 81:39–44

Guilford P, Prakash S, Zhu JM, Rikkerink E, Gardiner S, Bassett H, Forster R (1997) Microsatellites in *Malus* x *domestica* (apple): abundance, polymorphism and cultivar identification. Theor Appl Genet 94:249–254

Hardy OJ, Vekemans X (2002) SPAGEDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Mol Ecol Notes 2:618–620

Hokanson SC, Szewc-McFadden AK, Lamboy WF, McFerson JR (1998) Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus* x *domestica* Borkh. core subset collection. Theor Appl Genet 97:671–683

Hokanson SC, Lamboy WF, Szewc-McFadden AK, McFerson JR (2001) Microsatellite (SSR) variation in a collection of *Malus* (apple) species and hybrids. Euphytica 118:281–294

Horvath A, Balsemin E, Barbot JC, Christmann H, Manzano G, Reynet P, Laigret F, Mariette S (2011) Phenotypic variability and genetic structure in plum (*Prunus domestica* L.), cherry plum (*P. Cerasifera* Ehrh.) and sloe (*P. Spinosa* L.). Sci Hortic 129:283–293

Iketani H, Yamamoto T, Katayama H, Uematsu C, Mase N, Sato Y (2010) Introgression between native and prehistorically naturalized (archaeophytic) wild pear (*Pyrus* spp.) populations in Northern Tohoku, Northeast Japan. Cons Genet 11:115–126

INIA (2011) Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Inventario Nacional de Recursos Fitogenéticos. http://wwwx.inia.es/webcrf/CRFesp/Paginaprincipal.asp. Accessed 26 September 2011

Itoiz R (2000) Caracterización de la colección de manzanos (*Malus* x *domestica* Borkh) del banco de germoplasma de la Universidad Pública de Navarra. Evaluación de su variabilidad. Dissertation, Universidad Pública de Navarra

Jacobs MMJ, Smulders MJM, van den Berg RG, Vosman B (2011) What's in a name; genetic structure in *Solanum* section *Petota* studied using population-genetic tools. BMC Evol Biol 11:42

Janick J, Moore JN (1996) Fruit breeding. Wiley, Chichester

Jing RC, Vershinin A, Grzebyta J, Shaw P, Smykal P, Marshall D, Ambrose MJ, Ellis THN, Flavell AJ (2010) The genetic diversity and evolution of field pea (*Pisum*) studied by high throughput retrotransposon based insertion polymorphism (RBIP) marker analysis. BMC Evol Biol 10:44

Kenis K, Keulemans J (2005) Genetic linkage maps of two apple cultivars (*Malus* x *domestica* Borkh.) based on AFLP and microsatellite markers. Mol Breed 15:205–219

Larsen AS, Asmussen CB, Coart E, Olrik DC, Kjaer ED (2006) Hybridization and genetic variation in Danish populations of European crab apple (*Malus sylvestris*). Tree Genet Genomes 2:86–97

Laurens F, Durel CE, Lascostes M (2004) Molecular characterization of French local apple cultivars using SSRs. Proc XIth Eucarpia Symp Fruit Breed Genet 1 and 2:639–642

Liebhard R, Gianfranceschi L, Koller B, Ryder CD, Tarchini R, van de Weg E, Gessler C (2002) Development and characterisation of 140 new microsatellites in apple (*Malus* x *domestica* Borkh.). Mol Breed 10:217–241

Mariette S, Tavaud M, Arunyawat U, Capdeville G, Millan M, Salin F (2010) Population structure and genetic bottleneck in sweet cherry estimated with SSRs and the gametophytic self-incompatibility locus. BMC Genet 11:77

Meirmans PG, van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes 4:792–794

Miranda C, Urrestarazu J, Santesteban LG, Royo JB, Urbina V (2010) Genetic diversity and structure in a collection of ancient Spanish pear cultivars assessed by microsatellite markers. J Am Soc Hortic Sci 135:428–437

Morgante M, Rafalski A, Biddle P, Tingey S, Olivieri AM (1994) Genetic-mapping and variability of seven soybean simple sequence repeat loci. Genome 37:763–769

Noiton DAM, Alspach PA (1996) Founding clones, inbreeding, coancestry, and status number of modern apple cultivars. J Am Soc Hortic Sci 121:773–782

Pereira-Lorenzo S, Ramos-Cabrer AM, Díaz-Hernández MB (2007) Evaluation of genetic identity and variation of local apple cultivars (*Malus* x *domestica* Borkh.) from Spain using microsatellite markers. Genet Resour Crop Evol 54:405–429

Pereira-Lorenzo S, Ramos-Cabrer AM, Gonzalez-Diaz AJ, Diaz-Hernandez MB (2008) Genetic assessment of local apple cultivars from La Palma, Spain, using simple sequence repeats (SSRs). Sci Hortic 117:160–166

Pereira-Lorenzo S, Costa RML, Ramos-Cabrer AM, Ribeiro CAM, da Silva MFS, Manzano G, Barreneche T (2010) Variation in grafted European chestnut and hybrids by microsatellites reveals two main origins in the Iberian Peninsula. Tree Genet Genomes 6:701–715

Pereira-Lorenzo S, Costa RML, Ramos-Cabrer AM, Ciordia-Ara M, Ribeiro CAM, Borges O, Barreneche T (2011) Chestnut cultivar diversification process in the Iberian Peninsula, Canary Islands, and Azores. Genome 54:301–315

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Ramos-Cabrer AM, Pereira-Lorenzo S, Diaz-Hernandez MB (2007) Morphology and microsatellites in Spanish apple collections. J Hortic Sci Biotechnol 82:257–265

Richards CM, Volk GM, Reilley AA, Henk AD, Lockwood DR, Reeves PA, Forsline PL (2009) Genetic diversity and population structure in *Malus sieversii*, a wild progenitor species of domesticated apple. Tree Genet Genomes 5:339–347

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. Mol Ecol Notes 4:137–138

Santesteban LG, Miranda C, Royo JB (2009) Assessment of the genetic and phenotypic diversity maintained in apple core collections constructed by using either agro-morphologic or molecular marker data. Span J Agr Res 7:572–584

Schlötterer C (2004) The evolution of molecular markers: just a matter or fashion? Nat Rev Genet 5:63–69

Silfverberg-Dilworth E, Matasci CL, van de Weg WE, van Kaauwen MPW, Walser M, Kodde LP, Soglio V, Gianfranceschi L, Durel CE, Costa F, Yamamoto T, Koller B, Gessler C, Patocchi A (2006) Microsatellite markers spanning the apple (*Malus* x *domestica* Borkh.) genome. Tree Genet Genomes 2:202–224

Stöck M, Ustinova J, Lamatsch DK, Schartl M, Perrin N, Moritz C (2010) A vertebrate reproductive system involving three ploidy levels: hybrid origin of triploids in a contact zone of diploid and tetraploid palearctic green toads (*Bufo viridis* subgroup). Evolution 64:944–959

Tessier R, David J, This P, Boursiquot JM, Charrier A (1999) Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. Theor Appl Genet 98:171–177

Urrestarazu J, Miranda C, Santesteban LG, Royo JB (2010) Análisis comparativo de la diversidad genética retenida en las colecciones nucleares de los bancos de germoplasma de manzano del Noreste español. Actas de Horticultura 55:263–264

Urrestarazu J, Laquidáin MJ, Miranda C, Santesteban LG, Royo JB (2011) Comparative analysis of the genetic diversity maintained in Apple germplasm Banks from Northeastern Spain. Acta Hortic 918:655–660

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

van Treuren R, Kemp H, Ernsting G, Jongejans B, Houtman H, Visser L (2010) Microsatellite genotyping of apple (*Malus* x *domestica* Borkh.) genetic resources in the Netherlands: application in collection management and variety identification. Genet Resour Crop Evol 57:853–865

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M et al (2010) The genome of the domesticated apple (*Malus* x *domestica* Borkh.). Nature Genet 42:833–839

Volk GM, Richards CM, Henk AD, Reilley AA, Bassil NV, Postman JD (2006) Diversity of wild *Pyrus communis* based on microsatellite analyses. J Am Soc Hortic Sci 131:408–417

Volk GM, Richards CM, Henk AD, Reilley AA, Reeves PA, Forsline PL, Aldwinckle HS (2009) Capturing the diversity of wild *Malus orientalis* from Georgia, Armenia, Russia, and Turkey. J Am Soc Hortic Sci 134:453–459

Weissinger AK (1985) Technologies for germplasm conservation *Ex Situ*. In: Orians G, Brown G, Kunin W, Swierzbinski J (eds) The preservation and valuation of biological resources. University of Washington Press, Seattle, pp 3–32

World Apple and Pear Association (2010) European apple and pear crop forecast. World Apple and Pear Assn, Brussels, Belgium