Genetic Diversity of Hazelnut (*Corylus avellana* L.) Germplasm in Northeastern Spain

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Additional index words. filbert, genetic relationships, microsatellite, simple sequence repeat, SSR markers

Abstract. In Spain, hazelnut is mainly cultivated in Catalonia, a region in the northeast. The province of Tarragona accounts for 88% of the total Spanish area planted to hazelnut. Almost 80% of the production in Tarragona is of the local cultivar Negret, with others cultivated to a lesser extent. Minor cultivars are only sporadically present in older orchards, farm yards and gardens, and have been collected for preservation. In this work, 16 SSR markers were used to fingerprint 18 minor hazelnut cultivars from northeastern Spain. Their microsatellite profiles were combined with those of 15 Spanish cultivars characterized in a previous work, and used to study the genetic diversity in 33 genotypes including local Spanish germplasm. The SSR analysis allowed development of unique profiles of each of the 18 cultivars, and no new case of synonymy was detected. A high level of genetic diversity (mean $H_e = 0.7$) was observed in 33 genotypes, although a high number of them showed a close genetic relationship. The dendrogram generated by UPGMA cluster analysis placed the 33 accessions into nine main groups, related to their putative pedigrees or geographical area of cultivation. All investigated Negret-type cultivars were found to be distinct from Negret, and only a few cultivars within this germplasm appeared to be seedlings of Negret. The results will be useful in the conservation of hazelnut germplasm and in the selection of parents for use in breeding.

The European hazelnut (*Corylus avellana* L.) is one of the world's major nut crops. Its geographic distribution extends from the Mediterranean coast of North Africa northward to the British Isles and the Scandinavian Peninsula, and eastward to the Ural Mountains of Russia, the Caucasus Mountains, Iran, and Lebanon (Thompson et al., 1996). Total worldwide hazelnut production is fifth after that of cashew (*Anacardium occidentale* L.), almond [*Prunus dulcis* (Miller) D.A. Webb], walnut (*Juglans regia* L.), and chestnut (*Castanea* spp.). Turkey has long been the leading producer and exporter of hazelnuts, accounting for about 71% of world production. Italy is second with over 13%,

the United States third with 4.1%, and Spain fourth with 2.8%. Azerbaijan, Iran, Georgia, China, France, and Greece are other important producers (FAOSTAT, 2007).

In Spain, hazelnut is mainly cultivated in Catalonia, a region in the northeast. The province of Tarragona accounts for 88% of the total Spanish area planted to hazelnut (Fig. 1). Minor hazelnut-growing areas include Castellón, Asturias, País Vasco, Aragón, and Navarra. Orchards in Tarragona province have been classified into two topographic groups. The first group is of orchards of the inland mountain ranges of the province ("Priorat-Prades"), located on hilly slopes and characterized by a low level of mechanization and low nut yield (500–800 kg·ha⁻¹). The second group is of orchards in flat areas of the region called "Camp de Tarragona" located near the Mediterranean coast that use modern mechanized techniques and show high nut yield (2,000–2,500 kg·ha⁻¹). Most commercial production is from this second area (Tous, 2005). Almost 80% of Tarragona's production is of the native cultivar Negret. 'Negret' and 'Pauetet' are sold as the commercial type "negreta" that receives the best prices on the national markets. Other Spanish cultivars (Grifoll, Gironell, Morell, Culplà, Ribet, and Trenet) are cultivated to a lesser extent. A few Italian cultivars (i.e., San

Giovanni, Tonda di Giffoni, and Tonda Gentile Romana) were introduced because of their high commercial value and have shown good adaptation to the conditions of the area.

Modern agriculture requires high yield for profitability, and consequently the number of cultivars planted has declined in recent years. To avoid loss of local germplasm, efforts have been made on a worldwide scale to collect and preserve genetic diversity. In Tarragona, the diverse local hazelnut germplasm was investigated by Tasia Valls (1975), who assigned cultivars to three groups: I) one main cultivar distributed in all hazelnut cultivation areas of the province and in regular plantations; II) cultivars that are common in some areas of the province and in regular plantations; III) minor cultivars that are not cultivated in regular plantations but are occasionally found scattered in orchards (Table 1). This germplasm has been collected and preserved in collection fields (Köksal, 2000) to preserve the genetic variability for future use (Table 2).

Traditional methods to characterize and identify hazelnut cultivars are based on phenotypic observations (Thompson et al., 1978; UPOV, 1979), but this approach is subject to environmental influences and thus requires several years to correctly define the traits of a plant. Isozyme polymorphism was proposed in the 1980s and early 1990s as an alternative and more effective method for cultivar identification and studies of genetic relationships (Ahmad et al., 1987; Rovira, 1997; Solar et al., 1997). During the last decade, DNA markers have proven to be convenient for accurately identifying cultivars due to their high discriminating power at a relatively low cost. Among the available DNA markers, microsatellite or simple sequence repeat (SSR) markers appear to be best-suited to cultivar fingerprinting. They are generally codominant, highly polymorphic, highly reproducible, and permit exchange of results among different laboratories as well as construction of an integrated database. Microsatellite markers were recently developed in *C. avellana* and evaluated in seven other *Corylus* species by Bassil et al. (2005a, 2005b) and Boccacci et al. (2005). On the basis of their high level of polymorphism, the most interesting loci were used to fingerprint and to identify mistakes in hazelnut accessions from several germplasm repositories (Botta et al., 2005; Gökirmak et al., 2005). Moreover, results were used to verify synonymies and parentage hypotheses and to investigate genetic relationships among cultivars grown in important (Boccacci et al., 2006; Ghanbari et al., 2005) production areas. SSR loci were also placed in a genetic map, and some of them were found linked to a dominant allele for resistance to eastern filbert blight caused by *Anisogramma anomala* (Peck) E. Müller (Mehlenbacher et al., 2006).

In the present work, 18 minor hazelnut cultivars from northeastern Spain were DNA-typed using microsatellite markers. Their SSR profiles were combined with those of 15 Spanish cultivars characterized by

Received for publication 11 Oct. 2007. Accepted for publication 24 Jan. 2008.

The authors are grateful to Prof. Giovanni Me (Dipartimento di Colture Arboree, Torino, Italy) for his generous collaboration and Prof. Shawn A. Mehlenbacher (Oregon State University, Corvallis, OR) for reviewing the manuscript. The research was funded by Regione Piemonte Administration (Italy), INIA and MAPYA (Spain), and by the European Union project SAFENUT (AGRI GEN RES 068).

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Fig. 1. Regions of Tarragona province (northeastern Spain) with hazelnut cultivation areas shown in dark (from Tassias Valls, 1975, modified).

Boccacci et al. (2006) and used to study the genetic diversity in this local germplasm, including cultivars of international interest in cultivation and breeding.

Materials and methods

Plant material and DNA extraction. Leaves were sampled from 18 accessions (Table 3) conserved in the germplasm collection field of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) of Reus (Tarragona, Spain). Genomic DNA was extracted from 0.2 g of leaves in a Tris-EDTA-NaCl buffer containing 0.25 M NaCl, 0.2 M Tris, pH 7.6, 2.5% PVP 40,000, 0.05 M Na₂EDTA, and 0.1% β-mercaptoethanol, using a modified protocol described by Thomas et al. (1993).

PCR amplification and microsatellite analysis. Sixteen SSR loci were used: CaT-A114, CaT-B107, CaT-B501, CaT-B502, CaT-B503, CaT-B504, CaT-B505, CaT-B507, CaT-B508, CaT-B509, CaT-B511, CaT-C001 and CaT-C504 (Boccacci et al., 2005) and CaC-A102, CaC-B020, and CaC-B028 (Bassil et al., 2005a). PCR amplification was performed in a volume of 20 µL

containing 50 ng of DNA, 0.5 U of *Taq*-DNA polymerase (Ampli*Taq* Gold, Applied Biosystems, Inc., Foster City, CA), 2 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM MgCl₂, 200 µM dNTPs, and 0.5 µM of each primer. PCR conditions were as follows: an initial denaturation step at 95 °C for 9 min followed by 26 cycles of denaturation (30 s at 95 °C), annealing (45 s at 55 °C and 50 °C for CaT-B502), and extension (90 s at 72 °C). The final elongation step was at 72 °C for 45 min. The forward primers were labeled with a fluorochrome (6-FAM, HEX, NED, or PET), and amplification products were analyzed using an ABI Prism 377 sequencer (Applied Biosystems, Inc.). Results of the run were then processed with Genescan software and allele sizes were estimated using the GeneScan-500 LIZ size standard (Applied Biosystems).

Data analysis. Genetic relationships among 18 accessions analyzed in this study and 15 Spanish cultivars characterized by Boccacci et al. (2006) using the same afore-said methods (DNA extraction, PCR amplification, and SSR analysis), were investigated by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis.

Genetic distances (1000 bootstraps) were computed as $D = [1 - (\text{proportion of shared alleles})]$ using the program Microsat (Minch, 1997). Cluster analysis was performed using the Neighbor software in the Phylip v.3.5c package (Felsenstein, 1989), and a dendrogram was constructed using the TreeView program (Page, 1996). The software Identity 1.0 (Wagner and Sefc, 1999) was used to calculate expected (H_e) and observed (H_o) heterozygosities.

Results and Discussion

In a previous study, Boccacci et al. (2006) characterized and investigated the genetic relationships among 78 hazelnut cultivars, including 15 from Spain, using the same 16 SSR loci used in this study. We expanded the work to include 18 additional local cultivars from Tarragona province. In this paper, data from both studies were pooled for statistical analysis and investigation of genetic relationships among the 33 cultivars and genetic diversity in the local hazelnut germplasm.

The 16 SSR loci resulted in unique genotypic profiles for all of the 18 cultivars (Table

Table 1. Spanish hazelnut cultivars described and classified by Tasia Valls (1975).

Classification	Cultivar name	Presumed synonym names
Group I	Negret	
Group II	Culplà	
	Gironell	Constanti, Gironenca, Grossal de Constanti
	Grifoll	Queixal de gos
	Morell	Capellut, Flocal, Falsetana, Fort, Rojeta,
	Pauetet	
	Ribet	
	Trenet	
Group III	Artellet	Artell nano
	Apegalos	Artell
	Ametllenca	
	Castanyera	Castanyal, Castanyenca, Cul de Madrina, Grossal; Barcelona in USA; Fertile de Coutard in France
	Closca molla	Culplà moll, Mollar
	Gironenc	Vermellet
	Lluenta	
	Martorella	
	Negret capellut	Capellut
	Negret garrofi	Garrofina, Ralladet, Ratllada
	Punxenc	Negret caputxi, Negret garrofi
	Pinyolenc	Floquet
	Planeta	Culplà d'Alforja
	Queixal de llop	
	Queixal de ruc	
	Ratllada	Gironell d'Alforja
	Ratolí	Negret ratolí
	Ros	Ros de la Selva
	Rosset	Falsetà, Rosset de valls
	Sant Jaume	
	Sant Joan	
	Sant Pere	
	Vimbodí	

3), and no new cases of synonymy were detected (Fig. 2). Cases of synonymy were previously reported by Boccacci et al. (2006). The total number of alleles was 114, and the

range for each locus was from 5 to 10 (mean: 7.1). H_e ranged from 0.50 to 0.80 (mean: 0.70), while H_o ranged from 0.57 to 1.00 (mean: 0.80). These data indicate high

genetic diversity in the local germplasm, comparable to that found in the larger set of cultivars analyzed by Boccacci et al. (2006). The high level of heterozygosity is a consequence of the self-incompatibility of this species. All cultivars require pollen from other genotypes to produce nuts (Germain and Sarraquigne, 2004), with the exception of the partially self-compatible 'Tombul' and 'Montebello' (syn. 'Nocchione') (Mehlenbacher and Smith, 1991).

The dendrogram generated by UPGMA cluster analysis (Fig. 2) placed the 33 accessions into nine main groups (A to I).

The most important Spanish cultivar Negret, cultivated in regular orchards in all hazelnut cultivation areas of Tarragona, was placed adjacent to Pauetet, with which it appears to have a close relationship. The three cultivars in group I share at least one allele at each locus (Table 3 and Boccacci et al., 2006). The codominant Mendelian inheritance of SSR markers allows identification of possible parent-offspring pairs in a group of closely related cultivars. Pedigree reconstruction is also possible when two cultivars share one allele at all of the loci and may be linked by a parent-offspring relationship. 'Pauetet', also known as 'd'en Bardina', is considered an open-pollinated seedling selected and propagated from the Alcover area (Alt Camp, Fig. 1) for its good nut and kernel characteristics and productivity (Table 2). 'Vimbodí' was a seedling selected and cultivated by growers in the Vimbodí area (Conca de Barberà). 'Negret' is very common in Tarragona orchards, and

Table 2. Morphological characteristics of the hazelnut cultivars grown in Tarragona province, Spain.*

Cultivar	Vigor	Habitus	Suckering	Percent kernel		Fruit shape	Fruit size
					by wt		
Ametllenca	Weak	Semi-erect	Very strong	43		Short sub-cylindrical	Medium
Apegalos	Very weak	Semi-erect	Medium	42		Globular	Small
Artellet	Weak	Spreading	Weak	50		Globular	Small
Castanyera	Very vigorous	Semi-erect	Medium	43		Globular	Large
Closca Molla	Vigorous	Erect	Very strong	58		Globular	Medium
Culplà	Weak	Spreading	Medium	49		Globular	Small
Gironell	Very vigorous	Erect	Weak	43		Globular	Small
Grifoll	Vigorous	Erect	Medium	41		Long sub-cylindrical	Small
Lluenta	Vigorous	Spreading	Weak	47		Ovoid	Small
Martorella	Vigorous	Semi-erect	Strong	44		Long ovoid	Medium
Morell	Weak	Spreading	Very strong	44		Ovoid	Medium
Negret	Low	Semi-erect	Strong	49		Ovoid	Medium
Negret capellut	Weak	Spreading	Medium	44		Conical	Small
Negret garrofi	Weak	Semi-erect	Medium	51		Short sub-cylindrical	Medium
Pauetet	Vigorous	Erect	Medium	48		Ovoid	Medium
Pinyolenc	Medium	Erect	Very strong	42		Ovoid	Small
Planeta	Vigorous	Spreading	Medium	44		Globular compressed	Small
Punxenc	Medium	Very erect	Very strong	47		Ovoid	Large
Queixal de llop	Medium	Spreading	Very strong	43		Long sub-cylindrical	Medium
Queixal de ruc	Vigorous	Semi-erect	Strong	49		Long sub-cylindrical	Large
Ratllada	Weak	Erect	Medium	48		Globular	Medium
Ratolí	Medium	Erect	Very strong	53		Ovoid	Small
Ribet	Vigorous	Spreading	Very strong	47		Ovoid	Medium
Ros	Vigorous	Erect	Very strong	45		Globular	Medium
Rosset	Very weak	Spreading	Medium	48		Ovoid	Small
Sant Jaume	Weak	Spreading	Medium	41		Conical	Small
Sant Joan	Weak	Semi-erect	Weak	49		Long ovoid	Very small
Sant Pere	Medium	Erect	Very strong	46		Ovoid	Small
Trenet	Vigorous	Semi-erect	Medium	39		Globular	Small
Vermellet	Medium	Semi-erect	Medium	45		Ovoid	Medium
Vimbodí	Weak	Spreading	Strong	40		Short sub-cylindrical	Large

*The classification of the different characters was done following Thompson et al. (1978) and UPOV Guidelines (UPOV, 1979).

Table 3. Allele sizes (in bps) at 16 SSR loci in 18 hazelnut cultivars from Tarragona, Spain.^a

Cultivar	CaC-A102	CaC-B020	CaC-B028	CaT-A114	CaT-B107	CaT-B501	CaT-B502	CaT-B503	CaT-B504	CaT-B505	CaT-B507	CaT-B508	CaT-B509	CaT-B511	CaT-C001	CaT-C504
Ametllença	290	304	284	265	271	167	167	167	167	167	167	167	167	167	167	167
Apegalos	294	304	284	257	265	167	167	167	167	167	167	167	167	167	167	167
Artellet	290	304	286	259	259	161	161	161	161	161	161	161	161	161	161	161
Lluenta	304	304	284	259	261	169	171	171	171	171	171	171	171	171	171	171
Martorella	304	304	284	259	259	167	169	169	169	169	169	169	169	169	169	169
Negret	304	304	274	284	259	271	169	171	169	171	169	162	111	140	157	161
Negret capellut	294	304	284	271	271	167	171	171	171	171	171	171	171	171	171	171
Negret garrofi	288	304	278	284	257	157	157	157	157	157	157	157	157	157	157	157
Pinyolenc	304	304	276	284	261	167	167	167	167	167	167	167	167	167	167	167
Planeta	286	304	276	284	259	265	173	173	173	173	173	173	173	173	173	173
Punxenc	304	304	278	284	257	171	171	171	171	171	171	171	171	171	171	171
Ratlada	290	304	274	284	269	169	169	169	169	169	169	169	169	169	169	169
Ratolí	304	304	284	265	271	167	171	171	171	171	171	171	171	171	171	171
Ros	288	304	272	284	265	167	169	169	169	169	169	169	169	169	169	169
Rosset	296	304	284	265	265	167	171	171	171	171	171	171	171	171	171	171
Sant Jaume	290	304	276	284	257	261	—	—	—	—	—	—	—	—	—	—
Sant Joan	304	304	276	284	259	265	171	171	171	171	171	171	171	171	171	171
Sant Pere	290	304	274	284	259	259	167	171	171	171	171	171	171	171	171	171
Vimbodí	290	304	284	259	265	167	171	171	171	171	171	171	171	171	171	171

^aSSR profile of 'Negret' was reported by Boccacci et al. (2006). The alleles shared among 'Negret' and 'Negret capellut', 'Negret garrofi', 'Punxenc', 'Ratolí', and 'Vimbodí' are shown in bold; alleles that do not match are in italic.

thus it is likely that Negret is a parent of both above-mentioned cultivars. The presence of a common allele at the S-locus further supports the parent-offspring relationship between 'Negret' (S₁₀S₂₂) and 'Pauetet' (S₁₈S₂₂) (Mehlenbacher, 1997).

In several of the inland mountain areas of the province, growers distinguish among 'Negret' and some morphotypes such as 'Negret caputxi' (syn. 'Punxenc' in Figuerola, Alt Camp), 'Negret capellut', 'Negret garrofi', and 'Negret ratolí' (syn. 'Ratolí') whose growth habit and nut characteristics are similar to those of 'Negret' (Table 2). Our results indicate that they are distinct cultivars, confirming the classification of Tassias Valls (1975). They show different genetic profiles from each other and from 'Negret' and were placed in separate clusters in the dendrogram. Yet their DNA profiles show that 'Negret' shares at least one allele at all loci with 'Punxenc', at 14 loci with 'Ratolí', at 13 loci with 'Negret capellut', and at 12 loci with 'Negret garrofi' (Table 3). The alleles that failed to match in 'Ratolí' (CaT-B511 and CaT-C504), 'Negret capellut' (CaT-B107, CaT-B511, and CaT-C001), and 'Negret garrofi' (CaT-A114, CaT-B107, CaT-B502, and CaC-B028) show discrepancies of 2–6 bp from the respective alleles in 'Negret', while a 12-bp discrepancy was observed in 'Negret garrofi' at locus CaT-A114 (Table 3). Short mutations are consistent with the stepwise mutation model proposed for microsatellite evolution (Jarne and Lagoda, 1996) and have already been observed between parents and progeny, such as in grape (Bowers et al., 1999; Piljac et al., 2002; Vouillamoz et al., 2003) and in hazelnut (Boccacci et al., 2006). The hypothesis of Tassias Valls (1975) that Negret is a cultivar population appears to be valid only from a commercial point of view, as the commercially sold "negreta" mixture indeed includes not only Negret and Pauetet but also these Negret-like minor cultivars. Our SSR fingerprints as well as careful observation of morphological and phenological traits indicate that Negret and these Negret-like minor cultivars are indeed different. The future of Negret-like minor cultivars is uncertain because nurseries are now propagating only Negret. The minor Negret-like cultivars persist only in older orchards and farm yards.

The cultivars 'Grifoll', 'Gironell', 'Morell', 'Culplà', 'Ribet', and 'Trenet', cultivated in regular orchards but only in some areas of the province, were placed in separate clusters. 'Grifoll' shares at least one allele at each locus with 'Martorella' (Table 3 and Boccacci et al., 2006); the two form group G. 'Martorella', a variety known only in Alforja, has a leafing out and flowering time similar to 'Grifoll' which is commonly cultivated in that region. 'Gironell' is the main cultivar in Constantí area (Tarragonès), where it is also called 'Grossal' or 'Grossal de Constantí'. It was placed in group B with the minor cultivars 'Queixal de ruc', 'Castanyera' (syn. 'Barcelona'), 'Negret garrofi', 'Ametllença', and 'Punxenc'. Their presence

Fig. 2. UPGMA dendrogram of 33 Spanish hazelnut cultivars based on alleles at 16 SSR loci. *Cultivar analyzed by Bocacci et al. (2006) with 16 SSR loci.

is limited to some orchards in the main areas where 'Gironell' is cultivated (Alt Camp and Tarragonès) (Tasias Valls, 1975). According to the literature (Tasias Valls, 1975), 'Punxenc' is also known as 'Negret caputxi' in Figuerola (Alt Camp) and 'Negret garrofi' in Fatarella (Terra Alta); 'Negret garrofi' is considered a synonym of 'Ratlada'. Our results indicated that neither Punxenc nor Ratlada was a synonym of Negret garrofi because the three cultivars have unique genotypes (Table 3). 'Morell' and 'Culplà' are mainly cultivated in Vandellós (Baix Camp), Tivisa (La Ribera d'Ebre), and in the Priorat-Prades region. 'Culplà' is also cultivated in

Fatarella and Horta-Arnes (Terra Alta) in eastern Tarragona. In several areas, including Alforja, Culplà is named Planeta (syn. Culplà d'Alforja), while in Vilanova de Prades (Priorat-Prades) growers distinguish between Culplà moll and Culplà fort, indicating with these two denominations the cultivars more commonly known as Closca molla and Culplà, respectively. The different morphological characteristics of the Culplà cultivars (Table 2) and our SSR results (Table 3) did not confirm these suspected synonymies. 'Morell' was placed in group H with 'Queixal de llop', 'Rosset', and 'Negret capellut', all three of which are cultivated in the same

areas as 'Morell' (Tasias Valls, 1975). These cultivars have similar morphological traits (Table 2). All of them, except for 'Rosset', have long husks that often clasp the nuts and hinder their drop at maturity. This problem is particularly evident in 'Morell' and 'Negret capellut'; for this reason 'Morell' is called 'Capellut' in Camp de Tarragona. 'Culplà' was placed in group C with 'Ratlada', a variety grown in Alforja (Baix Camp) and Villalonga (Tarragonès) (Tasias Valls, 1975). 'Ratlada' is also called 'Gironell d'Alforja', but its SSR genotype showed it not to be a synonym of 'Gironell' (Table 3 and Bocacci et al., 2006). 'Ribet', the main variety cultivated in Alforja, was placed in group D with 'Ros', 'Closca molla', and 'Planeta', which were found by Tasias Valls (1975) in two areas (Baix Camp and Priorat-Prades) adjacent to the cultivation center of 'Ribet'. Finally, 'Trenet' is considered to have originated as a selected seedling in Valls (Alt Camp) and is widespread in the Alt Camp region where the other cultivars of group F are localized (Tasias Valls, 1975). Trenet appears to be closely related to Pinyolenc, with which it shares at least one allele at each locus, and also with Sant Joan, a cultivar native to Valls (Table 3 and Bocacci et al., 2006).

Minor cultivars not present in modern orchards but rather scattered in older Tarragona orchards (Table 1, Group III) were placed in all clusters of the dendrogram. They appear to be closely related to the major cultivars and, in some cases, appear to have a parent-offspring relation with them. According to Tasias Valls (1975), most cultivars of Tarragona province were selected from local wild populations and from open-pollinated seedlings. Moreover, he postulated that the center of origin of these minor varieties was the mountainous area of Camp de Tarragona, where they were propagated and then spread to nearby cultivation areas. It is possible that these minor cultivars are the progenitors of the main cultivars, although the opposite case may appear more likely. Further analyses of wild and domesticated hazelnuts, including chloroplast SSRs, would improve our understanding of the origin and diffusion of the local hazelnut germplasm.

In conclusion, the present study shows a high level of genetic diversity among hazelnut cultivars from Tarragona province, although several cultivars showed a genetically close relationship. These results may prove useful in the conservation of hazelnut germplasm and in the selection of parents for use in breeding. Finally, the SSR profiles will extend the database recently developed by Bocacci et al. (2006), a useful tool to investigate genetic relationships and to identify cultivars and synonyms.

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