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

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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 ‘Pauet’ 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 Ttasias Valls (1975), who assigned cultivars to three groups: I) one main cultivar distributed in all hazelnut cultivation areas of the province; 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 (Kökşal, 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; Göürkımak et al., 2005). Moreover, results were used to verify synonyms 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 Antiosemmia 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
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 Agroalimentaries (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% 3-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-B502, and CaC-B504 (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 (AmpliTag Gold, Applied Biosystems, Inc., Foster City, CA), 2 μL of 10X 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). 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 aforementioned 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 $D = \left(1 - \frac{\text{proportion of shared alleles}}{}\right)$ using the program Microsat (Minch, 1997). Cluster analysis was performed using the Neighbor software in the Phylib 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 Tasias Valls (1975).

| Classification | Cultivar name       | Presumed synonym names                       |
|---------------|---------------------|---------------------------------------------|
| Group I       | Negret              | Negret capellut, Negret capellut, Garrofina, Raylatad, Ratllada |
|               | Tulipa              | Castanyal, Castanyenca, Cull de Madrina, Grossal, Barcelona in USA; Fertile de Court in France |
|               | Gironell            | Gironella Moll, Gironenca, Grossal de Constanti |
|               | Morell              | Capellut, Flocal, Falsetana, Fort, Rojeta, Pauetet |
|               | Ribet               | Capellut |
|               | Treuet              | Mollar, Mollar |
| Group II      | Artellet            | Closca moll |
|               | Apegalos            | Gironena, Gironella, Grossal de Constanti, Grossal de Constanti |
|               | Artell llano        | Negret caputxi, Negret garrofii |
|               | Pinyolenc           | Floquet, Flaxet, Rosset de valls |
|               | Planeta             | Culpia d’Alforja |
|               | Queixal de llop     | Negret, Negret capellut, Negret capellut |
|               | Queixal de ruc      | Negret capellut, Negret capellut |
|               | Rallada             | Negret capellut, Negret capellut, Negret capellut |
|               | Rosol               | Negret capellut, Negret capellut, Negret capellut |
|               | Rosset              | Negret, Negret capellut, Negret capellut |
|               | Sant Joan           | Negret capellut, Negret capellut, Negret capellut |
|               | Sant Pere           | Negret capellut, Negret capellut, Negret capellut |
|               | Vimboh              | Negret capellut, Negret capellut, Negret capellut |

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

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

| Cultivar       | Vigor  | Habitus     | Suckering | Percent kernel by wt | Fruit shape by wt | Fruit size |
|---------------|--------|-------------|-----------|---------------------|-------------------|------------|
| Ametlenca     | Weak   | Semi-erect  | Very strong | 43                  | Short sub-cylindrical | Medium     |
| Apegalos      | Very weak | Semi-erect  | Medium | 42                  | Globular    | Small      |
| Artellet      | Weak   | Semi-erect  | Medium | 50                  | Globular    | Small      |
| Castanyera    | Very weak | Semi-erect  | Medium | 43                  | Globular    | Large      |
| Closca Moll   | Vigorous | Semi-erect  | Very strong | 58                  | Globular    | Medium     |
| Culpia        | Weak   | Semi-erect  | Medium | 49                  | Globular    | Small      |
| Gironell      | Very vigorous | Erect     | Very strong | 58                  | Globular    | Large      |
| Gironmenca    | Vigorous | Semi-erect  | Very strong | 47                  | Long ovoid  | Medium      |
| Llenta        | Vigorous | Semi-erect  | Strong  | 47                  | Ovoid      | Medium      |
| Martorella    | Vigorous | Semi-erect  | Strong  | 47                  | Ovoid      | Medium      |
| Morell        | Weak   | Semi-erect  | Very strong | 44                  | Ovoid      | Medium      |
| Negret        | Low    | Semi-erect  | Strong  | 44                  | Ovoid      | Medium      |
| Negret capellut | Weak  | Semi-erect  | Strong  | 44                  | Conical    | Small      |
| Negret garrofi | Weak  | Semi-erect  | Medium | 51                  | Short sub-cylindrical | Medium     |
| Pauetet       | Vigorous | Semi-erect  | Medium | 48                  | Short sub-cylindrical | Medium     |
| Pinyolenc     | Medium  | Erect      | Very strong | 42                  | Globular    | Small      |
| Planeta       | Vigorous | Semi-erect  | Medium | 44                  | Globular    | Small      |
| Punxenc       | Medium  | Very strong | Strong  | 47                  | Ovoid      | Large      |
| Queixal de llop | Medium  | Semi-erect  | Very strong | 43                  | Short sub-cylindrical | Medium     |
| Queixal de ruc | Vigorous | Semi-erect  | Strong  | 49                  | Long ovoid  | Medium      |
| Rallada       | Weak   | Semi-erect  | Very strong | 48                  | Globular    | Medium      |
| Rosol         | Medium  | Eect       | Very strong | 48                  | Globular    | Medium      |
| Rosset        | Very weak | Semi-erect  | Strong  | 41                  | Conical    | Small      |
| Sant Joan     | Weak   | Semi-erect  | Very strong | 49                  | Very small  | Small      |
| Sant Pere     | Medium  | Semi-erect  | Very strong | 46                  | Very small  | Small      |
| Treuet        | Vigorous | Semi-erect  | Medium | 39                  | Thin ovoid  | Small      |
| Vermboh       | Medium  | Semi-erect  | Medium | 45                  | Ovoid      | Medium      |

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

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 ‘Tombull’ and ‘Montebello’ (syn. ‘Nocchione’) (Mehlenbacher and Smith, 1991).

The dendrogram generated by UPGMA cluster analysis (Fig. 2) placed the 33 accesses 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). ‘Vimbodi’ was a seedling selected and cultivated by growers in the Vimbodi area (Conca de Barbera). ‘Negret’ is very common in Tarragona orchards, and
Table 3. Allele sizes (in bps) at 16 SSR loci in 18 hazelnut cultivars from Tarragona, Spain.

| Cultivar          | CaT-B107 | CaT-B114 | CaT-B106 | CaT-B501 | CaT-B502 | CaT-B503 | CaT-B504 | CaT-B505 | CaT-B506 | CaT-B507 | CaT-B508 | CaT-B509 | CaT-B511 | CaT-B512 | CaT-B500 |
|-------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Ametllenca        | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Artellet          | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Martorella         | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Negret garrofí    | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Planeta           | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Punxenc Ros       | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Sant Joan         | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |
| Vimbòz           | 125      | 154      | 118      | 124      | 184      | 194      | 160      | 160      | 111      | 111      | 140      | 154      | 150      | 150      | 161      |

of the province, growers distinguish among Negret and some morphotypes such as Negret caputxi (syn. Puxenc in Figuerola, Alt Camp), Negret capellut (Negret garrofí), 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 Tasias 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 Puxenc, at 14 loci with Ratolí, at 13 loci with Negret capellut, and at 12 loci with Negret garrofí (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 garrofí (CaT-A114, CaT-B107, CaT-B502, and CaT-B028) show discrepancies of 2–6 bp from the respective alleles in Negret, while a 12-bp discrepancy was observed in Negret garrofí 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 (Bocacci et al., 2006). The hypothesis of Tasias 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’, ‘Culpla’, ‘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 Bocacci 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 Constans area (Tarragonés), where it is also called ‘Grossal’ or Grossal de Constans. It was placed in group B with the minor cultivars ‘Queixal de ruc’, ‘Castanyera’ (syn. ‘Barcelona’), ‘Negret garrofí’, ‘Ametellenca’, and ‘Puxenc’. Their presence
is limited to some orchards in the main areas where ‘Gironell’ is cultivated (Alt Camp and Tarragona) (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 ‘Ratllada’. Our results indicated that neither Punxenc nor Ratllada was a synonym of Negret garrofi because the three cultivars have unique genotypes (Table 3). ‘Morell’ and ‘Culpla’ are mainly cultivated in Vandellòs (Baix Camp), Tivisa (La Ribera d’Ebre), and in the Priorat-Prades region. ‘Culpla’ is also cultivated in Fatarella and Horta-Arnes (Terra Alta) in eastern Tarragona. In several areas, including Alforgia, Culpla is named Planeta (syn. Culpla d’Alforja), while in Vilanova de Prades (Priorat-Prades) growers distinguish between Culpla moll and Culpla fort, indicating with these two denominations the cultivars more commonly known as Closoa moll and Culpla, respectively. The different morphological characteristics of the Culpla cultivars (Table 2) and our SSR results (Table 3) did not confirm these suspected synonyms. ‘Morell’ was placed in group H with ‘Queixal de Ilop’, ‘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. ‘Culpla’, was placed in group C with ‘Ratllada’, a variety grown in Alforja (Baix Camp) and Villalonga (Tarragona) (Tasias Valls, 1975). ‘Ratllada’ is also called ‘Gironell d’Aforja’, but its SSR genotype showed it not to be a synonym of ‘Gironell’ (Table 3 and Boccacci et al., 2006). ‘Ribet’, the main variety cultivated in Alforgia, was placed in group D with ‘Ros’, ‘Closoa moll’, 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 Boccacci 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 Boccacci et al. (2006), a useful tool to investigate genetic relationships and to identify cultivars and synonyms.

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