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RESEARCH ARTICLE

# Chloroplast SSR genetic diversity indicates a refuge for *Corylus avellana* in northern Portugal

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**Abstract** The genus *Corylus,* a member of the birch family Betulaceae, includes several species that are widely distributed throughout temperate regions of the Northern Hemisphere. The development of microsatellites or simple sequence repeats (SSRs) for non-coding regions of the chloroplast genome and their higher sequence variation compared with coding regions has provided a higher resolution tool for the study of cultivars and closely related taxa. Chloroplast polymorphisms provide a marker system to evaluate the genetic structure of plant populations. This study investigated genetic diversity in three cultivars and 32 genotypes of *Corylus avellana* L. from Portugal: 13 wild genotypes and 19 Portuguese landraces. Four of ten cpSSR loci were polymorphic, with diversity indices ranging from 0.111 to 0.244. Eleven chlorotypes were detected, and their relationships were analyzed using a network model. Haplotype A was most frequent in landraces and cultivars. Four chlorotypes (H, I, J and L) were found only in wild hazelnuts. The diversity of chlorotypes in the wild hazels, and the limited number reported in cultivars, suggests that northern Portugal was a refuge for hazel during the last ice age.

**Keywords** Chlorotype · *Corylus avellana* · Filbert · Hazelnut · Microsatellite

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## Introduction

Two different *Corylus* species are present in Europe: the European hazel, *C. avellana* L., which has a wide distribution, and the Turkish tree hazel, *C. colurna* L., restricted to the Balkans, Romania, and northern Turkey with isolated trees also reported in Iran and Georgia (Thompson et al. 1996). European hazel (*C. avellana*) is a member of the Betulaceae. The geographic distribution of European hazel extends from the Mediterranean coast of North Africa northward to the British Islands and the Scandinavian, and eastward to the Ural Mountains of Russia, the Caucasus Mountains, Iran, and Lebanon (Kasapligil

1972). Important cultivars in Europe and Turkey were selected over many centuries from local wild populations (Trotter 1921; Tasias Valls 1975; Thompson et al. 1996). Turkey has long been the world's leading producer and exporter of hazelnuts, accounting for about 71 % of world production (FAOstat 2008). Among trees nuts, European hazel is the fifth commercially most important after cashew (*Anacardium occidentale* L.), almond [*Prunus dulcis* (Miller) D. A. Webb], walnut (*Juglans regia* L.), coconut (*Cocos nucifera* L.) and chestnut (*Castanea* spp.). The use of hazelnuts dates to prehistoric times (Gokirmak et al. 2009). Hazelnuts are consumed as natural, blanched and roasted kernels, or as processed products including sliced, chopped kernels, hazelnut flour, oil and butter. In Portugal, hazelnut production has suffered a sharp decline since the 1990s, which saw the abandonment of its cultivation in favor of agronomic crops leading to the loss of local landrace. The number of landrace genotypes identified in Portugal is low and are denominated Molar, Grada de Viseu, Veiga and Comum (Silva et al. 2004). The loss of landrace genotypes can reduce the genetic diversity of residual populations, which is potentially catastrophic since genetic diversity is widely recognized as a key requirement for the long-term survival of species on an evolutionary time-scale. Genetic diversity provides the template for adaptation, evolution and survival of populations and species, especially in environments that are subject to climate change or to introduction of new pests, pathogens or competitors (Rajora and Mosseler 2001). Genetic analysis of populations requires suitable multivariate markers that can elucidate fine-scale details of spatial structure (Streiff et al. 1998) and reconstruct gene flow patterns (Streiff et al. 1999). Microsatellites or SSRs are highly polymorphic co-dominant markers generated by the polymerase chain reaction (PCR). Recently, nuclear SSRs were investigated in hazelnut and used for genotyping and phylogeny studies (Bassil et al. 2005; Boccacci et al. 2005, 2006; Gokirmak et al. 2009; Gürcan and Mehlenbacher 2010; Gürcan et al. 2010). Microsatellites also occur in the chloroplast genome of higher plants (Powell et al. 1995, 1996). These cpDNA microsatellites are usually single nucleotide repeats, mainly A–T repeats, and are highly polymorphic (Provan et al. 1999, 2001). In plants, cpSSRs have been widely employed in population and evolution studies and have proven to be particularly useful for deducing re-colonization routes and identification of hybridization events (Navascues and Emerson 2005; Boccacci and Botta 2009). Comparative analyses of nuclear and chloroplast microsatellites have become popular approaches because they can provide complementary and often contrasting information on genetic structure, differentiation and gene flow (pollen- and seed-mediated) within and among populations (Birky 1988; McCauley 1995; Ennos et al. 1999; Weising and Gardner 1999; Ishii et al. 2001; Lira et al. 2003; Ueno et al. 2005). Genetic diversity and differentiation measures derived from these markers can also support policies regarding conservation priorities for populations of threatened species (Petit et al. 1998). In angiosperms, chloroplast genomes are predominantly maternally inherited (mainly transmitted through the female gamete) and, thus, can reveal maternal lineages (Radetzky 1990; Rajora and Dancik, 1992; Birky 1995; Dumolin et al. 1995). This make them particularly sensitive to the effects of population fragmentation; partly because they have smaller effective population sizes than nuclear genomes and partly because seed-mediated gene dispersal is generally more limited than pollen-mediated gene flow (Pakkad et al. 2008). Consequently, chloroplast-specific markers are likely to provide good indicators of historical bottlenecks, founder effects and genetic drift (Li et al. 2007). In case of *C. avellana* chloroplast genomes inheritance is maternal. Since the number of landrace genotypes actually identified in Portugal was low, the aim of this work was to ascertain the level of maternal genetic diversity and differentiation of wild and landrace genotypes of *C. avellana*, by using cpSSR markers.

## Materials and methods

A total of 35 hazelnut accessions were used in this study: three cultivars, 19 landraces and 13 wild plants. 'Butler', 'Merveille de Bollwiller' and 'Longue d'Espagne' were included as controls. The 19 landrace genotypes were collected within an area of approximately 27,000 km$^2$, located between Northern and Central Portugal (latitude 40.30–41.70 and longitude 6.29–8.35) (Fig. 1). This area was recognized an area of hazelnut production in olden times (until 1980) (Galvão, 1968). The identification and location of these landrace genotypes was carried out by collecting

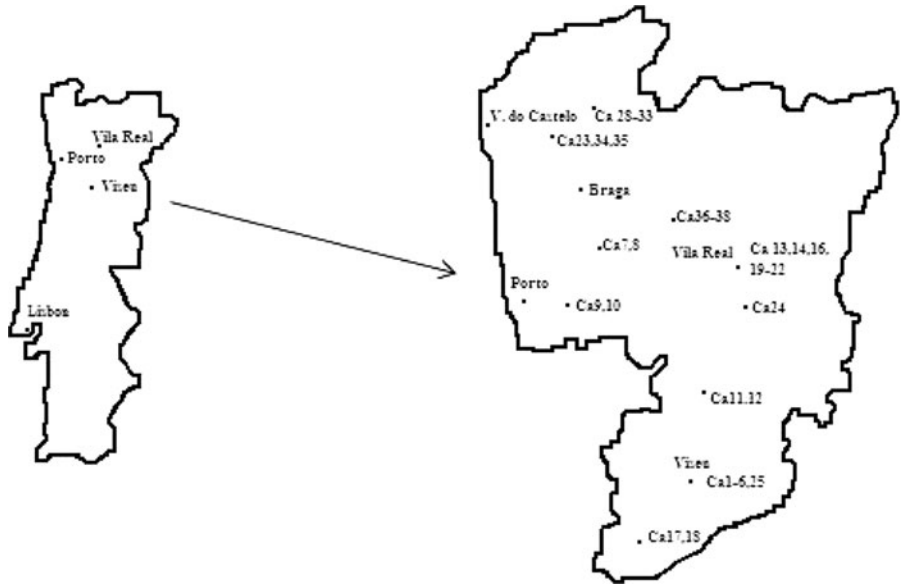


**Fig. 1** Map of northern Portugal showing sampling sites

oral testimonials from farmers and other older people in the region. Testimony confirmed that the landraces had existed in the region for >75 years (predating the World War II). Some of these accessions were previously included in the European Project Safenut (AGRI GEN RES 068). From each site we collected landrace genotypes showing morphological differences and appearing to be >75 years old. To avoid sampling related genotypes or resampling clones, all individuals selected were separated by at least 200 m. We searched for wild genotypes in natural and national parks with the collaboration of forest keepers who had knowledge of the existence and the location of the wild hazelnuts. Sampling in such parks was preferred due to their conservation value of the areas and because they are wild genotypes. Table 1 lists the accessions used in this study by group, and gives the geographical coordinates of each collection site. Immature catkins were collected in the field and kept cool during the transportation (about 2 h) and frozen in a −80 °C freezer. Total genomic DNA was extracted by using the a DNeasy kit (Qiagen) (Hilden, Germany), following the manufacturer's instructions. DNA concentration was determined by both spectrophotometry at 260 nm and gel electrophoretic analysis. The ten cpSSR loci in analysis comprised ccmp1, ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7 and ccmp10 (Weising and Gardner 1999), and cmcs1 and cmcs4 (Sebastiani et al. 2004) designed for *Nicotiana tabacum* L. and *Castanea sativa* Mill., respectively. PCR amplification was carried out with a reaction mixture (20 µl) consisting of 50 ng of template DNA, 10 µM of each primer, 200 µM dNTPs, 2 mM $MgCl_2$, 2 µl 10× $(NH_4)_2SO_4$ buffer and 1 unit of Taq DNA polymerase (Fermentas) (Germany). All cpSSR amplifications were performed under the following temperature cycling conditions: 3 min of denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C; and a final 20-min extension step at 72 °C. PCR products were separated on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, FosterCity, CA, USA). Results were processed with GeneMapper software and allele sizes (in base pairs, bp) were estimated from the GeneScan-350 ROX size standard (Applied Biosystems). The cpSSR data were analyzed with Popgene 1.32 software (Yeh et al. 1999). A chlorotype median network was constructed in the program Network v. 4.5 (Bandelt et al. 1999).

## Results

Of the ten pairs of chloroplast microsatellite primers, only four were polymorphic (Table 2). Locus ccmp2 showed four size variants, while ccmp4 showed three and ccmp3 and ccmp10 each showed two. Considering the allelic variants at the four loci, eleven different haplotypes were detected among the 35 genotypes and their relationships were analyzed under a median

**Table 1** Chlorotypes and origins of *C. avellana* genotypes studied

| Accession | Group | Haplotype | Region | Local | Longitude | Latitude |
|---|---|---|---|---|---|---|
| 1 | Landrace | A | Center | Viseu | 7°56′W | 40°39′N |
| 2 | Landrace | B | Center | Viseu | 7°56′W | 40°39′N |
| 3 | Landrace | A | Center | Viseu | 7°56′W | 40°39′N |
| 4 | Landrace | B | Center | Viseu | 7°48′W | 40°39′N |
| 5 | Landrace | D | Center | Viseu | 7°54′W | 40°39′N |
| 6 | Landrace | A | Center | Viseu | 7°49′W | 40°38′N |
| 7 | Landrace | B | Littoral | Felgueiras | 8°10′W | 41°22′N |
| 8 | Landrace | B | Littoral | Felgueiras | 8°10′W | 41°22′N |
| 9 | Landrace | C | Littoral | Castelo de Paiva | 8°16′W | 41°02′N |
| 10 | Landrace | A | Littoral | Castelo de Paiva | 8°16′W | 41°02′N |
| 11 | Landrace | E | Center | Moimenta da Beira | 7°34′W | 40°57′N |
| 12 | Landrace | A | Center | Moimenta da Beira | 7°34′W | 40°57′N |
| 13 | Cultivar | B | UTAD | Vila Real | 7°44′W | 41°17′N |
| 14 | Cultivar | B | UTAD | Vila Real | 7°44′W | 41°17′N |
| 16 | Cultivar | A | UTAD | Vila Real | 7°44′W | 41°17′N |
| 17 | Landrace | A | Center | Tondela | 8°04′W | 40°30′N |
| 18 | Landrace | B | Center | Tondela | 8°04′W | 40°30′N |
| 19 | Landrace | F | Inland | Vila Real | 7°45′W | 41°19′N |
| 20 | Landrace | A | Inland | Vila Real | 7°44′W | 41°19′N |
| 21 | Landrace | A | Inland | Vila Real | 7°44′W | 41°19′N |
| 22 | Landrace | G | Inland | Vila Real | 7°44′W | 41°19′N |
| 23 | Wild | H | Littoral | Ponte de Lima | 8°35′W | 41°44′N |
| 24 | Wild | A | Littoral | PNDI | 6°29′W | 40°30′N |
| 25 | Landrace | A | Center | Viseu | 7°48′W | 40°39′N |
| 28 | Wild | I | Littoral | PNPG | 8°15′W | 41°56′N |
| 29 | Wild | I | Littoral | PNPG | 8°15′W | 41°56′N |
| 30 | Wild | J | Littoral | PNPG | 8°15′W | 41°56′N |
| 31 | Wild | I | Littoral | PNPG | 8°22′W | 41°70′N |
| 32 | Wild | L | Littoral | PNPG | 8°22′W | 41°70′N |
| 33 | Wild | I | Littoral | PNPG | 8°22′W | 41°70′N |
| 34 | Wild | I | Littoral | Ponte de Lima | 8°35′W | 41°44′N |
| 35 | Wild | I | Littoral | Ponte de Lima | 8°35′W | 41°44′N |
| 36 | Wild | I | Inland | PNA | 7°49′W | 41°22′N |
| 37 | Wild | H | Inland | PNA | 7°49′W | 41°22′N |
| 38 | Wild | L | Inland | PNA | 7°49′W | 41°22′N |

network model (Fig. 2). The distribution of these haplotypes among the three different levels of human selection was not uniform (Table 3). Haplotype A was observed in nine landraces, one wild genotype, and in 'Merveille de Bollwiller'. Haplotype B was common in the landraces but was absent in the wild genotypes. Five rare haplotypes (C, D, E, F and G) were found in the landraces, but were absent in the wild genotypes and cultivars. One wild genotype from Peneda-Gerês National Park showed the rarest haplotype (J). The two main haplotypes (A and B) were present in the cultivars and landraces, and haplotype A was also present in one wild genotype. Haplotypes H, I, J and L were found only in wild genotypes north of the Douro River and were absent in the cultivars and landraces. Haplotype I was the most frequent in the wild genotypes. Chlorotype H was detected in two wild accessions, one from Ponte de Lima and one from

**Table 2** Eleven chlorotypes based on four polymorphic cpSSR loci. Allele sizes are in base pairs as determined by capillary electrophoresis

| Haplotype | Ccmp2 | Ccmp3 | Ccmp4 | Ccmp10 |
|---|---|---|---|---|
| A | 213 | 117 | 115 | 106 |
| B | 213 | 117 | 116 | 106 |
| C | 213 | 116 | 116 | 106 |
| D | 213 | 117 | 114 | 106 |
| E | 213 | 117 | 115 | 105 |
| F | 214 | 117 | 115 | 105 |
| G | 214 | 117 | 115 | 106 |
| H | 217 | 117 | 115 | 106 |
| I | 217 | 117 | 114 | 106 |
| J | 218 | 117 | 114 | 106 |
| L | 217 | 117 | 114 | 105 |

Six of ten cpSSR loci were monomorphic: Ccmp1 (129 bp), Ccmp5 (107 bp), Ccmp6 (97 bp), Ccmp7 (153 bp), Cmcs1 (102 bp) and Cmcs4 (107 bp)

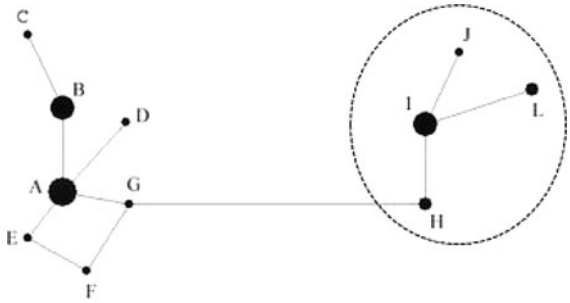


**Fig. 2** Median network of 11 haplotypes identified in 35 hazelnut genotypes. *Circle areas* are proportional to haplotype frequencies. The haplotypes within the *dashed circle* were only found in wild genotypes

Alvão Natural Park while haplotype L was found in two wild accessions, one from Peneda-Gerês National Park and another from Alvão Natural Park. Genetic diversity (H) values observed in the group of wild genotypes and landrace genotypes were 0.244 and 0.222, respectively. Diversity at the four polymorphic cpSSR loci was 0.359. Diversity within groups was 0.192, $G_{ST}$ was 0.425 and Nm was 0.676.

## Discussion

The ten cpSSR primer pairs were those used by Boccacci and Botta (2009) and included the six used by

**Table 3** Chlorotype number and frequency in 32 Portuguese *C. avellana* genotypes and three cultivars

| | Wild genotypes | Cultivated forms | | |
|---|---|---|---|---|
| | | Landrace genotypes | Cultivars | Total |
| Haplotype A | 1 (0.077) | 9 (0.474) | 1 (0.333) | 11(0.314) |
| Haplotype B | | 5 (0.263) | 2 (0.667) | 7 (0.200) |
| Haplotype C | | 1 (0.053) | | 1 (0.028) |
| Haplotype D | | 1 (0.053) | | 1 (0.028) |
| Haplotype E | | 1 (0.053) | | 1 (0.028) |
| Haplotype F | | 1 (0.053) | | 1 (0.028) |
| Haplotype G | | 1 (0.053) | | 1 (0.028) |
| Haplotype H | 2 (0.154) | | | 2 (0.057) |
| Haplotype I | 7 (0.538) | | | 7 (2.000) |
| Haplotype J | 1 (0.077) | | | 1 (0.028) |
| Haplotype L | 2 (0.154) | | | 2 (0.057) |
| N | 5 | 7 | 2 | 11 |
| H | 0.222 | 0.244 | 0.111 | 0.359 |

*n* number of samples, *N* number of haplotypes, *H* genetic diversity and chlorotype frequency shown in parentheses

Palmé and Vendramin (2002) in previous studies of hazelnut. Boccacci and Botta (2009) assessed diversity in cultivated forms (75 accessions) from Spain, Italy, Turkey and Iran, and found three sizes at ccmp2, 2 at ccmp3, 2 at ccmp4 and 2 at ccmp10 for a total of four haplotypes. Palmé and Vendramin (2002) found seven haplotypes in wild genotypes from natural forests in 11 European countries. We found the same number of size variants as Palmé and Vendramin (2002) at ccmp2 (3 sizes), ccmp3 (2 sizes) and ccmp10 (2 sizes). At ccmp4, we found the two sizes reported by Boccacci and Botta (2009) rather than just the one reported by Palmé and Vendramin (2002). In our work four polymorphic cpSSR primer pairs resulted in 11 haplotypes, four (J–L) exclusive to wild genotypes, five (C–G) exclusive to landraces, and two (A and B) shared haplotypes. The detection of exclusive haplotypes found in wild and landrace genotypes is probably due to geographic isolation and absence of plant improvement, respectively, and is clearly shown by the network diagram (Fig. 2). In fact, 13 genotypes from wild genotypes, collected from isolated forest sites in northern Portugal (Fig. 1) produced five different haplotypes, in contrast to the six haplotypes reported by Palmé and Vendramin (2002) who studied 248 wild genotypes from 26 natural populations across Europe.

This diversity in *C. avellana* in northern Portugal indicates that it was a refuge during the last ice age. Palmé and Vendramin (2002) excluded southern Italy and the Balkans as a source for post-glacial recolonization, as five haplotypes were identified in southeastern Europe but only two in western and northern Europe. The analysis of our samples with other molecular markers (nSSRs, ISSRs, AFLPs) also revealed high diversity. Tzedakis et al. (2002) and Petit et al. (2003) suggested that regions showing high levels of diversity could be identified as a potential refuge. Fossil pollen data (Huntley and Birks 1983) and phylogeographic studies (Hewitt 1996, 2000; Taberlet et al. 1998) show that in Europe the only areas that would have remained viable for the survival of temperate flora were the Iberian (Portugal and Spain), Italian and Balkan peninsula. Species like pine, oak and hazel appear to have been present in all three refuges (Taberlet 1998). Comparison of wild and landrace haplotypes showed clear differences. Haplotype B was not found in wild Portuguese hazelnuts, but is predominant in the landraces. Haplotype A is present in the landraces, but is rare in the wild genotypes. The existence of the Alvão, Marão, Peneda and Gerês mountains (1,200–1,600 m elevation with a north/northeast orientation) made gene flow more difficult in the southern direction. The presence of haplotype A in only one wild genotype prompts us to speculate that this accession from the Douro International Natural Park may be the ancestral haplotype for nine landraces. This wild genotype is situated on the rocky cliffs of the Douro river, which would allow easy seed dispersal by birds, small mammals or river flow. Seven of the nine landraces that share haplotype A with this wild genotype are located on the south bank of the Douro River, consistent with a possible ancestral relationship. The presence of natural barriers, like mountains, associated with empirical human selection can help explain differences in distribution between wild and landrace genotypes. It is believed that at the full extent of glacial advance, temperate flora survived in small pockets in environmentally favorable places and there were not broad expanses of trees and shrubs. The more extensive studies of wild and landrace hazels from other European countries would increase our understanding of haplotype distribution, and the origin and evolution of the European hazelnut.

We found a high level of chloroplast haplotype diversity in wild hazelnuts from a relatively small and restricted area. Information on genetic diversity in wild hazels will provide information relevant to diversity conservation as well as breeding. The unique haplotypes identified in wild genotypes suggests that those chlorotypes were excluded when superior plants were chosen from the local vegetation for clonal propagation. Additionally, the low number of wild individuals found in northern Portugal points to a need for short- and long-term conservation of genetic diversity.

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