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Castanea spp. biodiversity conservation: collection and characterization of the genetic diversity of an endangered species

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Abstract

Centuries of co-evolution between *Castanea* spp. biodiversity and human populations has resulted in the spread of rich and varied chestnut genetic diversity throughout most of the world, especially in mountainous and forested regions. Its plasticity and adaptability to different pedoclimates and the wide genetic variability of the species determined the spread of many different ecotypes and varieties in the wild. Throughout the centuries, man has used, selected and preserved these different genotypes, vegetatively propagating them by grafting, for many applications: fresh consumption, production of flour, animal nutrition, timber production, thereby actively contributing to the maintenance of the natural biodiversity of the species, and providing an excellent example of conservation horticulture.

Nonetheless, currently the genetic variability of the species is critically endangered and hundreds of ecotypes and varieties are at risk of being lost due to a number of phytosanitary problems (canker blight, *Chryphonectria parasitica*; ink disease, *Phytophthora* spp.; gall wasp, *Dryocosmus kuriphilus*), and because of the many years of decline and abandonment of chestnut cultivation, which resulted in the loss of the binomial male chestnut. Recently, several research and experimentation programmes have attempted to develop strategies for the conservation of chestnut biodiversity.

The purpose of this paper is to give an overview of the status of biodiversity conservation of the species and to present the results of a 7 year project aimed at the individuation and study of genetic diversity and conservation of *Castanea* spp. germplasm.

Key words: chestnut; germplasm identification; ex situ conservation; conservation and horticulture; Piedmont Region

Introduction

Fagaceae (*Cupuliferae*) includes eight genera (*Castanea, Castanopsis, Fagus, Lithocarpus, Nothofagus, Quercus, Trigonobalanis, Chrysolepis*) and about a 600-800 species. The genus *Castanea* is widespread in the Boreal Hemisphere (Fig.1) and includes 12 or 13 species according to classification (Table 1). The natural distribution of the European chestnut (*Castanea sativa*) includes Europe and all of the Mediterranean countries. In Asia (China, Korea, Japan, Vietnam) *C. crenata, C. mollissima, C. seguinii, C. henryi* occur. In North America, *C. dentata* is found between Ontario and Maine, along the Appalachian Mountain Range into Georgia and Alabama (Camus 1929) and *C. pumila* is found in the southeastern states.

All species are diploids ($x = 12$; $2n = 24$) (Jaynes 1962). The genus is taxonomically divided into 3 sections: *Castanea*, *Balanocastanon*, and *Hypocastanon*, but further revisions are expected (Johnson 1988) as a result of new genetic studies, which contest the validity of this classification (Santamour et al. 1986).

Castanea species biodiversity is very wide, reflecting the adaptation of the genus to different environmental conditions. It shows variability for morphological and ecological traits, vegetative and reproductive habits, nut size, wood characteristics, adaptability, and resistance to biotic and abiotic stresses, and the burden between natural biodiversity and human selection is very weak and sometimes unclear (Bounous 2002).

Species in the type section *Castanea*, which includes the most economically important species, display high genetic diversity. Different species are found in very different pedoclimates, but they prefer deep, soft, acidic soils (pH ranging from 4 to 6.5), temperate climates, and rainfall ranging from 700 to 1500 mm/year. The latitudinal distribution is related to altitude.

At low latitudes chestnut trees are found above 1500 m a.s.l., as on the slopes of Mount Etna in Italy (Polacco 1938), on the Sierra Nevada in Spain, and in Caucasus, where the species thrives at an elevation of 1800 m (Fenaroli 1945).

Tree shape and form are variable. *Castanea dentata* and *C. sativa* are upright, tall and slender trees, but some species have smaller size, round foliage and branches that start from the base. Other species are dwarf shrubs.

Co-evolution between *Castanea* spp. biodiversity and human populations has resulted in the spread of rich and varied chestnut genetic diversity throughout most of the world, especially in mountainous regions. Yet, currently, hundreds of ecotypes and varieties are at risk of being lost due to a number of phytosanitary problems (canker blight, *Chryphonectria parasitica*; ink disease, *Phytophthora* spp; gall wasp, *Dryocosmus kuriphilus*) and because of the many years of decline and abandonment of chestnut cultivation (Sartor et al. 2009).

The purpose of this paper is to give an overview of the biodiversity of the species, and to present the results of a 7-year project aimed at the individuation, description and conservation of *Castanea* spp. germplasm.

History and taxonomy

UPGMA analysis of isozyme-based genetic distance estimates (Dane et al. 2003) and phylogenetic analysis based on cpDNA sequence data (Lang et al. 2006) suggest that *Castanea* species are geographically structured. This is inconsistent with the current phylogeny based on cupule characteristics. The section *Castanea* appears to be paraphyletic with differentiation among species being best explained by their current geographical ranges. *C. crenata* appears to be the most basal taxa and sister to the remainder of the genus. The three Chinese species [*C. mollissima*, *C. seguinii* (*Castanea*) and *C. henryi* (*Hypocastanon*)] are supported as a single monophyletic clade and sister to a group containing the North American and European species. There appears to be weak but consistent support for a sister-group relationship between the North American species and European species.

The biogeographical history of the genus has been inferred from cpDNA data and molecular clock theory (Lang et al. 2007). A unique westward expansion of extant *Castanea* species has been hypothesized with *Castanea* originating in eastern Asia, followed by intercontinental dispersion and divergence between the Chinese and European/North American species during the middle Eocene, followed by subsequent divergence between the European and North American species during the late Eocene.

European species

Castanea sativa Mill. (European chestnut or sweet chestnut)

The genus *Castanea* appeared at the end of Miocene (15 million years ago) (Giordano 1993; Bounous 2001) and its indicators (*Cupuliferae* dissemination) include oak and beech. Leaves and one fossil chestnut resembling European chestnut dating back to 8.5 million years ago were found in Coiron Massif, France (Breisch 1995). During the quaternary era glaciations, chestnut trees receded southwards (at the end of Würmian glaciation).

In Europe, there were two taxa of chestnut: *C. sativa* and *C. latifolia* Sord. (Paganelli 1997). At the end of the last glaciation (Würmian), as pollen charts demonstrated, only *C. sativa* survived. *C. sativa* is now the only native species in Mediterranean and Central European regions.

In Europe, *C. sativa* is found from Turkey to Portugal and Spain. The Azores archipelago (25º - 31º W) is the most Occidental point for *C. sativa* and the Canary Islands is the most Southern point (27º - 29º N). Towards the north, chestnut fruit production reaches 52ºN latitude to the south of the United Kingdom, northern Germany, Poland and Ukraine.

It is found at sea level in some littoral areas of the northern Iberian peninsula, Calabria (39ºN, 16ºW) in Italy and Thessalia (Middle Eastern Greece, 39ºN, 22º10’W) and at up to 1100 m in the highest mountains of Trás-os-Montes (Northeast Portugal; 41ºN, 07ºW). However at lower latitudes the chestnut grows at up to 1500 m in Sierra Nevada (Granada, Southern Spain; 37ºN, 03ºW) and on the slopes of Etna in Sicily (37ºN, 14ºW) or even at up to 1800 m in the Caucasus Mountains (42ºN, 42ºW) (Bounous, 2002a; Pereira-Lorenzo et al. 2001; Gomes-Laranjo et al. 2007; Fernández De Ana Magán et al. 1997).

In South America, *C. sativa* was introduced into Chile by European settlers at the beginning of the 19^{th} century. It is mainly to be found along the Andes mountain range (34ºS, 69ºW – 41ºS, 72ºW) (Bounous et al. 2002a).

Castanea sativa is a tall tree of majestic appearance; it is vigorous and can exceed 30 m height and 400 years of age. Some century-old trees measure 6–7 m in girth. The nut (10–30 g) has a white–cream pulp and it can have pellicle intrusions into the kernel.

In Europe, the germplasm is very broad and the risk of genetic erosion is high, mostly in marginal or abandoned zones (Bounous et al. 2001; Pisani 1992). Conservation of the most interesting genetic materials, selected over centuries, is necessary to maintain valuable biodiversity. There are hundreds of cultivar names for chestnuts, many of which are synonyms or homonyms (Botta et al. 2001).

Asian Species

Castanea crenata Sieb. & Zucc. (Japanese chestnut). *Castanea crenata* can be dated by fossil findings to the middle of Jomon Civilization (1000–4000 BCE). From its zone of origin it spread from Japan to Korea and to Northeast China, and it was naturalized in South Korea and in Taiwan (Tanaka et al. 2005).

The species has been cultivated in Central and South Japan for 2000 years. It can be found between paddy fields and conifer forests, on fertile, recent volcanic soils. The species is distributed throughout the island (41ºN, 141ºW – 31ºN, 131ºW), but it is primarily cultivated at around 37ºN latitude and it is not found in the Okinawa region.

It prefers a mild summer climate, not too cold in winter, with high rainfall (1200–1400 mm/year) in summer. On the southern Japanese Islands, where there is abundant summer rain and mild winters, *C. crenata* grows to 1300 m a.s.l.. It is not as cold resistant as American and Chinese species (Rutter et al. 1991) and early flowering makes it sensitive to late spring frosts (Breisch 1995).

The tree does not normally exceed 8–10 m in height but can reach 15 m, and 60 cm in diameter. The adaxial side of leaves is dark-green and the abaxial side is light green. Leaves are acute with strongly marked edges, and leaf margins are crenate. Young leaves have scattered, disk-shaped trichomes and have long, protective, whitish pubescence on major veins (Camus 1929).

The nuts of *C. crenata* vary greatly from tree to tree; some are the largest in the genus and can weigh more than 30 g. The hilum scar is very wide and reaches the middle of the chestnut. They are not usually sweet, are sometimes astringent, and have an adherent pellicle, which is difficult to separate from the kernel (Tanaka and Kotobuki 1992).

In France, *C. crenata* germplasm has been widely used in breeding programs to obtain *Phytophthora*-resistant trees (Salesses et al. 1993).

Castanea mollissima Blume (Chinese Chestnut). This species owes its name to the thick pubescence on buds and on the abaxial side of the leaves. This is the most widespread native species in China. *C. mollissima* grows in sub-tropical, temperate-continental, and temperate-maritime regions with mild winters and hot summers where rainfall is about 1000 mm/year (mostly in the summer).

Chinese chestnut has been recently introduced into many countries for its plasticity and adaptability to different pedoclimates.

C. mollissima thrives from 41°29'N latitude in Jilin Province, close to Korea, to 18°31'N latitude North of Hainan Island. It grows in Hebei and Shandong, in the Yangtze Valley, from west to east and in Sichuan, Hubei, Anhui, Jiangsu and, in the southwest, in Yunnan Province, close to the Vietnamese border. *C. mollissima*, along with *C. crenata*, is also found in Korea: the former in northern Korea (40ºN, 126ºW) while the latter is more frequently found in the southern Korean Peninsula (36ºN, 127ºW), which is the main production area (Kim et al. 2005). It grows from 50 to 2800 m a.s.l. in a wide range of climatic conditions.

Many varieties and local ecotypes have been described, of which about 50 are cultivated. They are divided into six groups with different morphological, physiological, horticultural, and geographical features. Interesting germplasm includes plants with burs that turn red in early autumn, plants with hanging branches, and some very precocious dwarf types (Liu 1993).

C. mollissima is a medium-sized tree: 12 m tall and with trunk diameters of up to 75–80 cm. Leaf serrations are large, irregular, not well pronounced, and have a hairy, mucronate point. The adaxial leaf surface is bright green, the abaxial surface is whitish-grey or velvet due to pubescence.

The nuts are round or elliptical and show a long torch (the tip of the nut, formed by the remains of the styles) covered by a thick, white-cream pubescence; the pulp is very sweet, but not as sweet as the American chestnut, and it is richer in proteins than the Japanese and European species. The hilum scar is wide but less developed than in *C. crenata*. Chestnuts show thin, easy-to-peel pellicles (not invading the kernel); kernels are sweet and ripen early. In the Northern regions, chestnuts are small (< 15 g), show bright colour, and have a good, sweet taste. In subtropical regions, the nuts of most cultivars are large (15–20 g) with high starch content.

Castanea seguinii Dode. This small tree or shrub is scattered in subtropical regions and in southwestern China. The very small nuts (2–4 g) are harvested for nourishment by rural people. Trees are periodically coppiced to produce firewood. They have early flowering and continue to flower throughout the bearing season until frost (Bounous 2001). Other genotypes, coming from Jiangsu province, show shoots with 10-20 burs. The reflowering feature appears to be regulated by two recessive genes and early flowering depends on one dominant gene (Jaynes 1962). Genetic diversity has been studied through isoenzymes (Huang and Norton 1992) with the aim of finding compatible genotypes to produce dwarf rootstocks**.**

Castanea davidii Dode. Some authors consider *C. davidii* a variety of *C. seguinii* based on the many affinities.

Castanea henryi (Skan) Rehd. & E.H. Wils. Known as the willow leaved chestnut, or pearl chestnut, the species is native to the warm temperate subtropical climates of China. It grows along the Yangtze River Valley and in southern regions. It is cultivated for timber in Fujian and Zhejiang provinces.

C. henryi is a forest species that grows rapidly with upright (slender) trunk, over 30 m tall. The chestnuts (one per bur) are small (3–6 g) and marketed to some extent.

North American Species

Castanea dentata (Marsh.) Borkh. (American Chestnut). *Castanea dentata* grew in Long Island 30,000–50,000 years ago, as evidenced by pollen dating back to the last inter-glacial periods. It is native to the eastern United States and Canada and it spread from Ontario and Maine (on the Appalachian Range, 47ºN, 66ºW – 32ºN, 87ºW) to Georgia and Alabama, where it was long a dominant species. Its natural range once covered more than 200 million acres from the Canadian border to the Gulf of Mexico. (Rosengarten 1984). It grows rapidly, with an upright, slender trunk that can exceed 30 m in height and has a diameter of 3 m or more.

The destruction of *C. dentata* by canker blight, *Cryphonectria parasitica*, was the greatest disaster in the history of forest pathology (Roane et al. 1986; Anagnostakis 1987). The canker, first identified in New York in 1904 at the Bronx Zoological Park, led to the complete removal of the species from the forest canopy. West of the native range it is possible to find adult trees that escaped the blight.

C. dentata is the most cold resistant species of the genus. Northern zone genotypes can survive to -35°C (Ashworth 1964).

Stems are small, sharp, brown and hairless. Leaves are similar in shape and dimension to *C. sativa*, and are generally hairless, sometimes having just a few hairs on the mid-vein, and thin. Nuts are sweet, not astringent, and very small with a thin pellicle which is easily removed from the kernel.

Castanea pumila (L.) Mill. This polymorphic species is divided into two botanical varieties: *C. pumila* var. *pumila* (Allegheny chinkapin) and *C. pumila* var. *ozarkensis* (Ashe) Tucker (Ozark chinkapin) (Johnson 1987). Other authors include *C. floridana* and *C. alnifolia* into *C. pumila*. It is native in the United States from the east and southeast to the Ozark mountains of Arkansas and to Missouri and Oklahoma (Camus 1929). Chinkapin tree shapes can be bushy (*pumila*), creeping (with some reported to be stoloniferous) or 20 m tall (*ozarkensis*) (Pardo 1978; Johnson 1988).

A high variability of leaf form, size and colour has been observed in the same plant. Burs are small (1–5 cm in diameter) with soft thorns. They remain on the branches and contain a single nut, sometimes remaining all winter long. These sweet and tasty chestnuts are very small (1 g).

Castanea floridana Ashe (Sarg.). This is a decorative bushy plant native to the southeastern United States from Florida to Texas, where it is known as Florida chinkapin. It can be 6–7 m high. The nuts (one per bur) are very small, and the plants flower much later in the season than *C. pumila*.

Castanea ashei (Sudw.) Ashe. Ashe chinkapin is a 6–7 m tall tree scattered throughout North Carolina, Georgia and Florida.

Castanea alnifolia Nutt. Shrub or creeping chinkapin is a creeping shrub (30–60 cm) originating in southern United States, from Alabama to Florida.

Castanea paucispina Ashe. The distribution area of this creeping shrub (30–60 cm) includes Texas and Louisiana.

Materials and Methods

Within the framework of different research projects begun at the beginning of the 21^{st} Century, the individuation, study of genetic diversity and conservation of *Castanea* spp. germplasm is still in progress at the Department of Arboriculture of the University of Turin (Italy). The aim of the research is to collect, identify, describe and preserve ex situ the germplasm of *Castanea* spp. in order to provide additional strategies to complement current efforts to protect the species.

Through collaboration with many international research Institutions, to date more than 300 ancient trees, representative of chestnut genetic variability, have been individuated in different areas of Europe, Asia and USA. Each tree was numbered and localized by GPS in order to obtain a detailed cartography and genetic material (scions) was collected from each tree in order to realize a collection field in North West Italy.

Morphological and phenological observations

Leaves, flowers and nuts of analyzed cultivars were sampled in situ and ex situ in the collection field. Through direct observation, or published information, when available, phenological (bud break, times of male and female flowering, harvesting date) and morphological traits (shape of the leaf; morphology of the flower; nut size; nut stripes; nut colour through colorimetric analysis; % of double fruits; kernel quality; pellicle adhesion) were used to characterize the genotypes (Breviglieri 1955; Bounous 2002a; Beccaro et al. 2005; Beccaro et al., 2009).

Nut descriptors are very useful for understanding the presumed traditional uses of a variety, but phenological and morphological observations are not considered effective enough for genotype identification, being subject to environmental and developmental factors, and they may not be enough to individuate homonymous and synonymous among the different genotypes. Therefore the accessions are characterized both by phenological and morphological analysis conducted in situ and ex situ and by microsatellite markers (SSR).

Molecular analysis

Samples of young fresh leaves are collected in spring for molecular analysis; DNA analysis is successfully performed by multiplex analysis of seven microsatellite loci isolated in *Castanea sativa* (Marinoni et al. 2003; Botta et al. 2001) and *Quercus petraea* (Steinkellner et al. 1997; Casasoli et al. 2006; Goulao et al. 2001; Marinoni et al. 2003; Buck et al. 2003; Yamamoto et al. 2003): CsCAT-1, CsCAT-8, CsCAT-14, CsCAT-15, CsCAT-16, CsCAT-17, and CsCAT-41. Loci were chosen based on the ease of allele scoring, multiplexing ability, and their linkage group assignment (Barreneche et al., 2004). PCR is performed in 20 μl reaction volumes containing 50 ng of DNA, 0.5 Units of Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2 μl of GeneAmp 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 1 μl of 10% Bovine Albumin Serum (BSA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM labelled forward primer and 0.5 μM reverse primer. The forward primers are labelled with a fluorochrome (6-FAM, HEX or NED).

Samples are analysed on an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, Calif., USA). Data is processed by the GeneMapper Software 4.0 (Applied Biosystems) and alleles defined by their size (in bp), compared with the standard, GeneScan-500 LIZ.

Genetic relationships among the accessions were investigated by UPGMA cluster analysis.

Ex situ conservation

The realization of a germplasm collection field of chestnut genetic diversity is in progress. Vegetal material is collected from each mother-tree and grafted into a collection field (3 plants for each accession) located at the Chestnut Regional Repository in Chiusa Pesio, Cuneo Province (North Western Italy), on the border with France (44º 19' N; 7º 40' E; 575 m a.s.l.). The area has a typical temperate climate and is largely given over to chestnut culture. It is in the phytoclimatic transition zone of “cold *Castanetum*” and “hot *Fagetum*”, according to Mayr-Pavari’s classification (De Philippis 1937) which identifies five phytoclimatic zones by means of the dominant tree species.

Soils are made of river deposits, with a high concentration of sand and the actual soil depth (between 30-60 cm) is limited because of coarse gravel (Tani et al. 2007). Currently 5 hectares are given over to the *Castanea* arboretum. The collection field was created with an 8x11.50 m planting layout, in order to use the arboretum both for scientific research and for landscape and tourist activities.

Results & Discussion

Morphological and phenological observations

The main passport data for 105 ecotypes and varieties are shown in Table 2. Very high variability within the same genotype and among different genotypes is observed. Ecotypes and varieties are classified in longistaminate (e.g. Solenga), mesostaminate, brachistaminate (e.g. Pelosa) and astaminate (e.g. Neirana, Marrone and Gioviasca), depending on the catkin flower morphology. Morphological observations of leaves (Fig. 2) and flowers (Fig. 3) as well as phenological observations do not yield relevant differences that were able to separate one genotype from the others. Nut descriptors are also not particularly effective in discriminating among the accessions (Table 4 and Fig. 6). However, very wide biodiversity is observed among the different genotypes (Fig. 4). One of the most important nut descriptors is the mono-embryonic *versus* poly-embryonic. Mono-embryonic nuts were named “marroni” type in Italy in the Middle Ages. Genotypes producing poly-embryonic nuts were named chestnut type. In Europe, most genotypes produce mono-embryonic nuts and some of them are “marroni” type (Breisch 1995; Bounous 2001; Pereira-Lorenzo et al. 2006).

In the past, many botanists tried to describe chestnut genotypes through the definition of morphological descriptors. However, because of the plasticity of the species, a morphological descriptor able to separate each different variety from another has not been individuated.

The botanist Pier Antonio Micheli (1679-1737) (*Enumeratio rariorum plantarum (Manoscr.)*, (cited by Breviglieri, 1955) was the first to describe chestnut varieties based on their bur, fruit, leaf and flower characteristics. Later, about 300 varieties were classified by region and harvesting time in Italy Piccioli (1922), and Remondino (1926) referenced nearly 1000 denominations, many of them synonymous. Breviglieri (1955) established the base descriptors for chestnuts that are used today. Solignat and Chapa (1975) described French cultivars, and classified them into main or local interest. Those descriptions were updated by Bergougnoux et al. (1978) but focused on clonal selection of the main cultivars, and included hybrids used in the new plantations mixed with European chestnut, as in Cevennes.

Though the cultivation of chestnuts in Switzerland has mostly been abandoned, some cultivars were localised and described by Conedera et al. (1993), Conedera et al. (2004) and Gobbin et al. (2007).

In Spain, Elorrieta (1949) was the first to report on Spanish cultivars. In 1996, the first inventory for Galician cultivars in North-western Spain was published, presenting the variability and a description of the local varieties (Pereira-Lorenzo et al. 1996a and 1996b; Pereira-Lorenzo et al. 2006). These studies were extended to other important areas for nut production such as Asturias, Castile and León, Extremadura, Andalucía and the Canary Islands (Pereira-Lorenzo et al., 2001; Ramos et al. 2005).

Molecular analysis

The reading of the profiles is clear most of the time and the amplification of all loci is always well balanced, with peaks of approximately the same height. So far, the most polymorphic loci have been CsCAT1, CsCAT16 and QpZAG110. The number of alleles totaled 59 and ranged from 6 to 11 per locus, with an average of 8.4. The loci with high numbers of alleles were CsCAT1 (10 alleles) and CsCAT41 (11 alleles). Expected heterozygosity (He) averaged 0.77 and ranged from 0.65 (for CsCAT15) to 0.85 (for CsCAT17), while observed heterozygosity (Ho) averaged

0.89 and ranged from 0.73 (for CsCAT16) to 1.00 (for CsCAT17). The estimated frequency of null alleles (r) was a positive value for locus CsCAT 16 (0.019). The total probability of identity at all loci was 2.99 x 10^{-8}, thus cultivars with identical genotypes were considered synonyms.

The results obtained in our set of accessions showed that microsatellite loci detected considerable polymorphism and confirmed that these markers are suitable for fingerprinting chestnut cultivars. The polymorphism and discriminant power of each locus were evaluated on the basis of number of alleles, expected and observed heterozygosity (Table 3). The mean values of these parameters were comparable to obtained in chestnut by Marinoni et al. (2003) and Gobbin et al. (2007). All loci analysed are highly polymorphic and thus particularly suitable for DNA typing of chestnut cultivars.

The combined profiles across the 7 SSR loci show the presence of a total of 105 different genotypes: many synonymous varieties were found, especially among the Italian ecotypes and varieties of Marroni (e.g. Marrone di Gemonio, Marrone di Roccamonfina, Marrone di Castel del Rio, Marrone Caprese Michelangelo, Marrone di San Mauro Saline, Marrone di Segni, Marrone Val Susa and Marrone di Zocca have the same genetic profile). This indicates the existence of substantial genetic uniformity within some cultivated varieties but also the possibility of a polyclonal origin for some of the cultivated varieties; a second option is that individual species showing a genotype different from the norm may belong to another variety or ecotype, which has not yet been analysed, and are simply cases of misnaming.

Cluster analysis performed for the 36 most important accessions produced an UPGMA dendrogram depicting the genetic relationships within the studied accessions (Fig. 5). Gabiana and Travisò accessions were grouped in cluster I. Almost all accessions named Marrone clustered whit most Italian cultivars in the large cluster II. The interesting data observed was the existence of an unique genetic profile among these Marroni accessions that are cultivated in different Italian regions. Finally, all Euro-Japanese hybrids analysed, except Bouche de Bétizac, were placed in group III together with some *C. sativa* accessions.

Various studies (Botta et al. 2001, Marinoni et al. 2003) have demonstrated the usefulness of microsatellite (SSR) analysis in discriminating between genotypes. Both approaches - morphological and molecular - are considered useful in characterising some aspects of the germplasm (Beccaro et al. 2009), however differing information is given from the results.

Ex situ conservation

The DNA results confirm that in one site alone a huge amount of still to be implemented *Castanea* spp. Biodiversity has been amassed. To date, more than 300 *Castanea* spp. trees have been grafted into the arboretum with accessions coming from all over the world. The arboretum currently includes several European ecotypes and cultivars from Italy, Portugal, Spain (including the Canary Islands), France and Switzerland, and accessions from the U.S.A., China, and Japan. The collection also includes 17 Euro-Japanese hybrids (*C. sativa* x *C. crenata*) obtained from different countries. The majority of hybrids were obtained from in France in the '80s, by INRA (Institut National de la Récherche Agronomique) in order to create ink disease and canker blight resistant genotypes (Bounous et al. 2001) and are actually used both on "their own roots" or as rootstocks for superior varieties. On the one hand, hybridization increases the commercial viability of the species, thus increasing the amount of material grown and ''safeguarding'' *Castanea* spp. germplasm taken in general. However, it also has the potentially negative effect of genetically contaminating native populations (coppices).

According to the convention on biological diversity (CBD) signed in 1992, which gave ownership of biodiversity to national governments and required equitable benefit sharing for commercial use of biodiversity with the country of origin, the aim of the arboretum is to promote *Castanea* conservation by putting a value on biodiversity. As the arboretum will give the next generation of researchers a new opportunity to further assess the genetic variability of the species, genetic material will be provided to growers and nurseries on request, in order to improve the role of horticulture in the conservation of resources.

Future plans include increasing the number of genotypes in the arboretum, including important areas still not represented, such as Turkey and Greece, and establishing networks with other germplasm collections, e.g. the National Chestnut Germplasm Repository of China (Li Guo-tian et al. 2009).

Conclusions

In the past, the high horticultural value of many *Castanea* spp genotypes allowed the biodiversity of the species to be maintained by the human populations, representing an excellent case for the intersection of conservation and horticulture. However, today all the *Castanea s*pecies have serious conservation problems and despite the current efforts to protect them, their genetic variability is critically endangered.

The creation of the arboretum and similar initiatives carried out by other international Institutes represent the first step in stopping the loss of biodiversity; however, more research efforts are still needed to fully implement the different strategies already applied in the conservation of *Castanea* species.

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