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Genetic and morphological characterization of chestnut (*Castanea sativa* Mill.) germplasm in Piedmont (north-western Italy)

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Abstract

Castanea sativa Mill. is an important multipurpose tree species for north-western Italy, and specially for Piedmont Region. The preservation of its germplasm from the genetic erosion due to the changes in socio-economic structure of rural areas and specific pathogen attacks is critical. The principal aims of this work were to characterize the chestnut germplasm grown in Piedmont and investigate its genetic structure. Sixty-eight grafted chestnut trees were evaluated using 10 SSRs (simple sequence repeats) loci and 20 morphological descriptors.

Thirty-six different genotypes were identified; the analysis of the genetic structure of this germplasm revealed that four gene pools contributed to the formation of the population sampled . In general, cultivars tended to group into a main gene pool on the basis of their prevalent use and growing area. These results are substantially in agreement with those of the cluster analysis that was carried out to estimate the genetic relationships among the cultivars.

Morphological analyses showed large variation of traits among the individuals, related with the market destination of the nuts and useful for cultivar and clonal selection. Discriminant analysis was applied to find a correlation between genetic and morphological data: nut and leaf shape, nut hairness and male flower type resulted to be the most discriminant traits associated with the genetic structure.

In the end, this work clarified the genetic structure of the cultivated germplasm in Piedmont describing the main cultivars of the Region, giving useful information for conservation and breeding purposes.

Key words: cultivar identification, morphological traits, simple sequence repeat (SSR), genetic structure.

Introduction

The European or sweet chestnut (*Castanea sativa* Mill.) is an important tree species, with a invaluable historical and cultural heritage, that play an important role in the economic and environmental context of mountain areas.

In Italy the spread of chestnut has promoted the evolution of a rich varietal heritage in different pedoclimatic areas. During its expansion this species generated large populations different for many traits, relating to the fruit traits and to plant resistance to biotic and abiotic stresses; nowadays over 300 cultivars ('chestnut' and 'marrone') are described (Bounous 2002). Piedmont, a north-western Region of Italy, hosts a reach chestnut germplasm, including minor, often endangered, cultivars. In this Region the chestnut cultivation has a very wide distribution and involves worldwide known cultivars such as 'Marrone'.

The preservation of this germplasm from the genetic erosion due to the changes in socio-economic structure of rural areas and specific pathogen attacks (Arnaud et al. 1997; Bruneton 1984; Sartor et al. 2009) is an important objective in the agro-biodiversity conservation strategy (CBD 2002). Chestnut conservation is very important to save valuable genotypes, because they may retain special adaptative and technological traits and so meet the demands of the market that nowadays requires more and more typical products of superior quality (Negri 2003). Moreover, from a socio-economic point of view, chestnut can play an important role in promoting local identity and social cohesion as well as helping to preserve the landscape; where the cultivation of this species is well established, it has the potential to form the basis of initiatives that can be developed for the benefit of the local communities.

The conservation of this wide germplasm is considered problematic not only for its high level of genetic diversity, but also for the presence of numerous homonyms and synonyms with consequent confusion in the plant names (Bartolini et al. 1998; Beccaro et al. 2004; Ertan 2007; Gobbin et al. 2007). The traditional characterization of chestnut populations is based on morphological and agronomic traits. MacKey (1988) pointed out the importance of morphological traits in taxonomic

studies of cultivated plants. A great number of chestnut cultivars was described by morphological evaluation (Breviglieri 1951; Ertan et al. 2007; Lavialle 1906; Vigiani 1908). Nowadays, the progress in molecular biology techniques offers new powerful tools allowing conservation and protection of the genetic resources. Recently the most used molecular markers for the identification and characterization of chestnut germplasm are microsatellites or SSRs (Simple Sequence Repeats, Botta et al. 1999; Botta et al. 2001; Buck et al. 2003; Gobbin et al. 2007; Marinoni et al. 2003; Martin et al. 2009; Yamamoto et al. 2003).

This work was carried out in the frame of the European Project MANCHEST, aimed at selecting and characterizing chestnut cultivars grown or endangered in Piedmont by DNA typing, morphological traits description, chemical and sensory analysis. In this paper the results of genetic and morphological analysis are presented.

Materials and Methods

Plant material

Young leaves of 68 *C. sativa* individuals were collected in different Valleys in Piedmont, north-western Italy (Table 1) and labelled with the cultivar name and a code. All trees were grafted and were sampled from the canopy; 37 different cultivar names were recorded.

DNA extraction and SSR loci amplification

DNA was extracted from young leaves (0.2g) following the procedure described by Thomas et al. (1993), with minor modifications.

Samples were analysed at 10 SSR loci: CsCAT1, CsCAT3, CsCAT4, CsCAT6, CsCAT16, CsCAT17 (Marinoni et al. 2003) and EMCs15 (Buck et al. 2003) developed from *Castanea sativa*; QpZAG110 and QpZAG119 (Steinkellner et al. 1997) and QrZAG96 (Kampfer et al. 1998) developed from *Quercus petraea* and *Quercus robur*, respectively. Eight out of the 10 loci were mapped in different linkage groups (Barreneche et al. 2004). Orthology between *Quercus* and

Castanea genera was previously assessed (Akkak et al. 2010; Barreneche et al. 2004; Boccacci et al. 2004) showing that loci QpZAG110, QpZAG119 and QrZAG96 are conserved in chestnut and thus are suitable for fingerprinting and population genetic studies.

Samples were then analysed on an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, Calif., USA). Data were processed by the GeneMapper Software 4.0 (Applied Biosystems) and alleles defined by their size (in bp), compared with a standard (GeneScan-350 ROX, Applied Biosystems).

Morphological characterization

Nuts, leaves and inflorescences were sampled from each of the 68 individuals. The morphological analysis was performed on 25 fruits, 20 leaves and 20 inflorescences per tree.

The majority of descriptors (Table 2) were selected from the descriptor list for chestnut of the International Union for the Protection of New Varieties of Plants (UPOV 1989) and of the Inventory of Chestnut Research Germplasm and References (Bounous et al. 2002). Further descriptors were selected from Bolvanský and Mendel (2001).

Statistical analyses

Genetical analyses were performed after removing synonyms. Microsatellite data obtained at 10 SSR loci were processed using the software Identity 4.0 (Wagner and Sefc 2004) to calculate: allele frequencies, number of alleles, observed and expected heterozygosity (Nei 1973), the probability of identity (Paetkau et al. 1995) and the total paternity exclusion probability (Weir 1996). Deviation from Hardy-Weinberg equilibrium, excess and deficiency of heterozygotes, were tested using the program Genepop (Raymond and Rousset 1995).

To assess the genetic structure in the group of cultivars analysed, a model-based Bayesian procedure, as implemented in the program Structure (Pritchard et al. 2000), was used. This model ensure that the incidence of each cultivar in the original population may be calculated (Breton et al.

2008). The admixture model was applied and allele frequencies were assumed to be correlated. 10 trials of 20^5 Monte Carlo Markov Chain (MCMC) replications, following an introduction period (burn-in) of 10^5 repeats for each hypothesis, were used. More recently, it has been suggested that a better estimator of *K*, the number of homogeneous gene pools of origin for the populations studied, is the modal value of ΔK (Evanno et al. 2005). The statistic ΔK was calculated by Structure Harvester software (Earl et al. 2011) and used to selected the optimal *K* value.

Genetic relationships were investigated by UPGMA (Unweighted Pair Group Method) cluster analysis using the Statistica software (Stat Soft Inc. 1993). Genetic distances (1000 bootstraps) were computed as D= (1-proportion of shared alleles) by Microsat software (Minch 1997).

Multivariate analysis was carried out on morphological data. Discriminant analysis was performed on the standardized variables using Statgraphics software (http://www.statgraphics.com/). The analysis was elaborated considering all characteristics of the nuts, leaves and inflorescences shown in table 2, except for "ripening time" and "nut size" because these descriptors are more susceptible to the environment influence. The colour was detected according to the colorimeter Minolta coordinates (L*a*b*), instead of the visual scale, because this method gives more objective data. The initial classification criterion used was the gene pools identified by Structure program. The contribution of each variables to the classification was estimated by the standardized discriminant coefficient (Afifi and Clark 1984).

Results

Microsatellite variability and cultivar characterization

In order to characterize the informativeness of the 10 SSR loci for chestnut identification, the variability of each locus was assessed across the genotypes.

A total of 80 alleles was detected and the number of alleles per locus ranged from 4 (EMCs 15) to 14 (CsCAT6), with an average of 8.0 alleles per locus. This value was higher than the 7.4 alleles per locus found by Martin et al. (2010) using 7 SSR loci on 94 Italian accessions, but it was lower

than the values found for chestnut cultivars in Switzerland (9.75 alleles per locus) using 8 SSR on 164 individuals (Gobbin et al. 2007), in southern Spain (8.7 alleles per locus) using 7 SSR loci on 100 grafted chestnuts (Martin et al. 2009), and in Spain and Portugal (11.8 alleles per locus) using 10 SSR loci on 574 *C. sativa* accessions (Pereira-Lorenzo et al. 2010).

Allele frequencies ranged from 0.014 to 0.583; 22 (~27,5%) out of the 80 alleles detected had a particularly low frequency (0.014) and in most cases they were specific of a single genotype (Online Resource 1).

All loci were in Hardy-Weinberg equilibrium ($\alpha < 0.05$). Observed heterozigosity (Ho) values varied from 0.64 (EMCs 15) to 0.89 (CsCAT6), with an average of 0.75; expected heterozigosity (He) ranged from 0.59 (QrZAG96) to 0.83 (CsCAT6), with an average of 0.72. These values were comparable to those found by Martin et al. (2010), analyzing Italian chestnut cultivars. An excess of heterozygotes was significant ($\alpha < 0.05$) at CsCAT17 ($P = 0.038$). On the contrary, no loci showed a significant deficit of heterozygotes. The estimated frequency of null alleles showed positive values for 2 loci and precisely for CsCAT3 (0.047) and QpZAG110 (0.024), neverthless the number of studied samples was too small to draw conclusions about the occurrence of null alleles, as their presence can be only truly ascertained by studying their segregation or their frequency in a large population (Callen et al. 1993).

The ability of genetic markers to study pollen flow is represented by the paternity exclusion probability, which is the parameter used to describe the chance of correctly identifying pollen donors (Tanaka et al. 1999). The total paternity exclusion probability was 0.999; this index was high for CsCAT6 (0.661), with a mean value of 0.502 (range: 0.353-0.661).

The probability of identity (PI) for each locus ranged from 0.051 for CsCAT6 to 0.213 for QrZAG96 (mean= 0.122), whereas the total probability of identity was 2.96×10^{-10}. The highest discriminative power was shown by loci CsCAT3 and CsCAT6 (20 genotypes) and CsCAT1 (15 genotypes). The least informative locus was QrZAG96 with only 7 genotypes (Table 3).

The combination of profiles across all loci resulted in 36 different genotypes: 13 genotypes included 2 or more plants, while 23 genotypes were represented by single individuals with a unique genetic profile (Table 4). Microsatellite analysis identified four cases of synonymy (shown in Italic in Table 4) and six cases of homonymy (indicated in Table 4 with different numbers). Each different genotype was indicated with a cultivar name and a number was used to distinguish homonymous cultivars: hereafter these plants will be considered as true-to-type and the 36 cultivar names will be used without further mentioning the tree code. The genetic profiles of the 36 genotypes analyzed at 10 SSR loci are reported in Online Resources 2.

As reported by Pereira-Lorenzo et al. (2011) for chestnut, by Boccacci et al. (2006) for hazelnut, and by Díaz-Losada et al. (2010) in grapevine, genotypes are considered related by hybridization when they share at least one allele per SSR locus. In this paper 41 possible first degree relationships were found between the 27 genotypes, with more than 1 possible alternative for 22 genotypes.

Genetic structure

In order to investigate the population structure in the chestnut germplasm spread all over the Piedmont Region and assign individuals to different gene pools based on the genotypes, a model-Based Bayesian procedure, as implemented in the software Structure (Pritchard et al. 2000) was applied. This approach estimates the most likely number of clusters (K), or homogeneous gene pools, which have originated the present population; the estimate of K was based on ΔK, according to Evanno et al. (2005). A sharp signal was found at $K = 4$, thus indicating that four gene pools shaped the genetic structure of the population analysed. To check the composition of each population and each individual with respect to each population, further analysis was therefore carried out based on K=4. The final proportion of each of the four hypothetical gene pools present in each cultivar was obtained and the results are shown in Fig 1. The assignation of a cultivar to a specific gene pool was provided by a membership probability of *qi* (the mean proportion of

ancestry). Genotypes with a membership probability lower than 70% were considered to belong to more than one gene pool.

Twenty-nine genotypes (81%) showed a strong component derived from one specific gene pool, while only 7 genotypes (19%), resulted from different groups ('Pugnenga 1', 'Selvaschina', 'Precoce di Brignola', 'Ciapastra 2', 'Gabbiana 2', 'Neirana 2', 'Primemura').

In particular, the red gene pool included the Italian important cultivar 'Marrone' and the cultivars known as "Marrone-like" such as 'Garrone Nero' and 'Garrone Rosso'. The green gene pool included most cultivars from the south-eastern part of Piedmont, such as 'Frattona' and 'Gabbiana 1', suitable for dried chestnut and flour production. The blue gene pool included most of the cultivars grown in western Piedmont (Val Pellice). The yellow gene pool was constituted by samples coming from all parts of Piedmont.

The genetic relationships among the 36 genotypes are shown in a dendrogram obtained using UPGMA as clustering method (Fig. 2). The robustness of the nodes of the dendrogram was assessed with bootstrap analysis using 1000 iterations. The dendrogram separated the 36 genotypes into three main clusters A, B (B1, B2) and C. These clusters or sub-clusters revealed the red, green and blue gene pool identified by Structure software. The individuals of the yellow gene pool resulted dispersed across the dendrogram and 2 genotypes ('Pelosa' and 'Neirana 2') were set apart to form cluster A. Cluster B was divided in two sub-groups B1 and B2. The sub-group B1 included most genotypes of cultivars grown for the production of dried chestnut and flour (green gene pool); the sub-group B2 included the cultivars from the western Piedmont (blue gene pool) together with 'Madonna' and 'Servai d'l'oca' from the yellow gene pool. Finally, group C included the genotypes of the red gene pool, together with 'Solenca 2' and 'Primemura' (yellow gene pool).

Morphological traits

Morphological observations were carried out on the 68 *C. sativa* individuals and are reported in Online Resources 3a and 3b; since unique genotypes were 36, data for the individuals sharing the same genotype are presented as a range.

Discriminant analysis (Fig. 3) was applied to find a correlation between genetic and morphological data and point out the most discriminant morphological traits among all traits observed. The analysis was conducted using the gene pool identified by Structure as a classification criterion; only the samples (59) assigned to a specific gene pool (with an inferred ancestry >70%) were considered for the analysis. The first two discriminant functions explained 93,5% of the total variation. The value of correct classification of samples to the four genetic pools, used as grouping variable, was 98%. The variables that had the strongest effect on the discriminant functions were nut width/height ratio, nut hairiness, foliar blade length/width ratio and male flower type.

Discussion

Microsatellite variability and cultivar characterization

Our set of 10 SSR loci proved to have an high discriminative power (total probability of identity: 2.96×10^{-10}) for the investigated cultivars, so it is therefore highly unlikely to detect false synonyms with these loci, and it is also shown that it could be useful in parentage studies even when both parental individuals are unknown (total probability of paternity exclusion: 0.9999). At last, twenty-eight percent of the alleles detected were typical of a single genotype, underlining that the genetic richness of a germplasm can be present either in the form of allelic variability or of allelic "uniqueness" of some populations (Petit et al. 1998).

Thirty-six different genotypes were detected in the Piedmont germplasm. When more clones were analysed, the results highlighted a genetic intra-cultivar homogeneity for some of the most valuable cultivars such as 'Marrone', 'Garrone Rosso', 'Garrone Nero' and 'Gentile'. Over many centuries humans have influenced *C. sativa* populations. The cultivars which provided high quality nuts and/or timber (e.g. 'Marrone' and 'Garrone') were selected by growers and spread all over the

country through propagation and trading of plant material from different geographic areas. As stated by Pereira-Lorenzo et al. (2011) clonality depends largely on the importance of the cultivar within a region and it represents a low-risk strategy for maintaining local populations and the fittest genotypes within a population. The name 'Marrone' appeared for the first time in the manuscript "*Liber ruralium commodorum*", by the agronomist Pier de' Crescenzi, dated approx 1305, as 'Marrone di Milano'; in the last decade of 1300 in "*Tacuinum sanitatis*" by Giovannino de' Grassi, the 'Marrone' cultivars grown in Lombardia Region (Brianza) are praised for their high nut quality. Over time, the cultivar 'Marrone' is mentioned in all Italian chestnut growing areas (Bounous 2002). It is evident from the present research that the 'Marrone' cultivars studied in Piedmont have a monoclonal origin and were spread in the Region for the high nut quality; they maintained the name 'Marrone' but were identified by a geographical indication.

In the history of chestnut cultivation, the reduction of diversity produced by grafting may have been compensated by the use of seedlings as reported by Auge and Brandl (1997), Forneck (2005), Pereira-Lorenzo (2010). Hybridization could therefore have played an important role in the diversification process (Pereira-Lorenzo et al. 2011) and could explain the great diversity found in a small geographic area as Piedmont. It is also possible that a seedling of a renowed cultivar has been selected by growers for its superior traits or that nuts of the best varieties were used for multiplication, in both cases yielding new cultivars. The presence of 41 possible first degree relationships between 27 genotypes may suggest parentage relationships. These are very likely between cultivars such as 'Garrone rosso' and 'Garrone nero', and between cultivars suitable for flour production such as 'Gaggia' and 'Martiniana'. Yet, considering the number of loci analysed and the occurrence of multiple parentage alternatives, any conclusion would not be reliable without further analyses. In addition, in order to demonstrate parentage, the shared alleles would have to be identical by descent, meaning that they are recently descended from a single ancestral allele and not simply identical by state, which can happen by chance (Vouillamoz and Grando, 2006).

Cultivar denomination mistakes or misunderstands may have occurred in the long period of chestnut domestication and the subsequent abandonment of its cultivation in the Region. A poor specific literature and the level of oral divulgation have also contributed to increase mistakes (Gobbin et al. 2007). In addition, traditional cultivars are often named according to geographic origin, ripening period and traits of the nut, making their classification very difficult. For instance, the name 'Tempuriva', means "early ripening", and it is given by growers to local cultivars displaying an early fruit ripening, but not necessarily sharing other characters. The cultivars named 'Pelosa' are well known in Piedmont for the good nut size and high yield and form a heterogeneous group having in common only the presence of hairiness on the epicarp of the nut, as suggested by their name (pelosa = hairy). Lastly, 'Neirana', which is a cultivar characterized by a timber with excellent technological properties, is so called only for the blackish brown colour of the epicarp; the two 'Neirana' ('Neirana 1' and 'Neirana 2') individuals analyzed in this study were genetically different and even not related by hybridization.

Finally, 23 cultivars showed unique genotypes. These local cultivars are sometimes neglected, often endangered, and in some cases are represented by a single individual, such as in the case of 'Precoce di Brignola'. These plants should be considered valuable genetic resources, so they should be regarded as additional local source of genetic diversity which need to be maintained and protected.

Genetic structure

The genetic diversity of a species is the sum of genetic information within a gene pool. Thus, a clear understanding of the genetic structure within a gene pool is an important goal in the strategies of germplasm conservation and breeding programs. In this study the genetic structure of 36 chestnut accessions grown in Piedmont Region was investigated. The estimation of statistics revealed four 'gene pools' as the number of inferred populations from which the studied germplasm derives; the most precise interpretation of this value is that four homogeneous gene pools contributed to the

population sampled. The majority of accessions showed a strong component derived from a single gene pool, demonstrated by a high inferred ancestry value (Fig. 1).

In general, cultivars tended to group into a main gene pool on the basis of their prevalent use and growing area. The cultivar grown in south-western Piedmont, having in common the use (fresh and candying) grouped together in the red gene pool; cultivars grown in the south-eastern part of Piedmont (suitable for flour production) were included in the green gene pool, while most cultivars coming from western Piedmont formed the blue gene pool. The yellow gene pool comprised accessions of different geographical areas. These results are substantially in agreement with those of the cluster analysis.

The genetic differentiation of the south-eastern germplasm, confirmed by all different analysis approaches, could be due to gene flow and exchange of material across the Appennine chain with the neighbourhood Liguria Region where, several chestnut cultivars, including some named 'Gabbiana' and 'Siria', are cultivated to produce dried nuts and flour. Liguria, which extends along the Mediterranean coast, in the past was an important Region for trade by sea and therefore open to great material exchange with other Mediterranean areas; moreover ancient trails which crossed the mountains to the north, connecting inland areas to the sea (such as the salt routes running between Liguria and Piedmont, Liguria and Lombardy) could have played an important role in the movement of crop material such as grape (Torello Marinoni et al. 2009) and chestnut.

Morphological traits

Morphological characterization revealed phenotypic diversity in the evaluated traits. In Italy chestnut harvest is carried out from the beginning of September until mid-November, in a similar way as in Spain (Pereira-Lorenzo et al. 2006). The cultivars with an early ripening time are scattered in all gene pools identified by Structure, except in the green one. These cultivars, such as 'Madonna' and Tempuriva', are very interesting because they get better price on the market; moreover since *C. sativa* accessions tend to be harvested later than Asian species or euro-japanese

hybrids, usually characterized by nuts of lower quality, early nut ripening associated to high quality production, could be a useful genetic trait for breeding.

A large nut size, as showed for example by cultivars of the red gene pool such as 'Garrone Rosso' and 'Marrone', is desirable from the standpoint of harvesting, handling, fresh marketing and candying ("marrons glacés"). Instead, in most semi-processed and processed uses there is less emphasis on size given that the nuts can be easily mechanically peeled. In northern Italy small sized nuts such as those of the green gene pool are very appreciated for the production of flour and dried nuts ('white chestnuts'). On the contrary, in Spain, small nuts have a low market value and for this reason this trait is considered negative and its removal is a priority in breeding projects (Pereira-Lorenzo et al. 2006).

A bright brown pericarp with darker stripes and a sub-rectangular shape is an appreciated trait for the fresh market because consumers identify these traits with good quality (Solar et al. 2005).

Further appreciable qualities of chestnut are a low percentage of epysperm intrusion in the kernel and monoembriony, both important traits for marketing. Low pellicle intrusion and monoembriony allow an easy pellicle removal for processing and in particular for the production of confectioneries requiring a whole seed. Indeed for the most part of cultivars grown in Piedmont (94%) the seed coat penetration was not much prominent or was even absent, as also reported by Bolvanský and Mendel (2001) for French, Spanish and other Italian cultivars. Few cultivars (19%) had no or low percentage of double seeds, while 61% of varieties had very high presence of double seeds (>12%) unlike what was found in Spain, where relatively few accessions (only up to 25%, depending on region) had the detrimental character of producing divided nuts, as reported by Pereira-Lorenzo et al. (2006).

Concerning the leaf traits, two shapes of leaves were observed; in particular, the lanceolate shape was typical of cultivars belonging to the red gene pool. The same gene pool was also characterized by cultivars with astaminate catkins, that do not produce pollen. To know the male flower type is very important for planting new orchards, because only longistaminate catkins produce abundant

pollen. In Piedmont 39% of the studied genotypes had astaminate catkins and 28% longistaminate ones, unlike what happens in Spain, where longistaminate catkins are the most frequent type (43% of total accessions), while astaminate ones are the least frequent (8%). Clonal variation of the male flower type (mesostaminate/longistaminate) was found in 'Ciapastra 1', 'Gabbiana 1', 'Siria'.

Finally, the discriminant analysis was able to correctly assign 98% of samples to the gene pools. The morphological traits that contributed to a larger extent to construct the discriminant function were related to nut hairiness, to nut and leaf shape, and to male flower type. Nut hairiness is a typical traits that can distinguish some Piedmont cultivars, to the extent that some of them are named 'Pelosa'. Nut shape is considered typical of a cultivar, although some variation exists due to environmental factors and rate of nut set within the burr: the importance of this trait for distinguishing cultivars in the Spanish germplasm was already highlighted by Pereira et al. (1996, 2006). The importance of pomological characteristics in differentiating accessions of different regions was also emphasized by Ertan et al. (2007). In addition, these authors underline the importance of male catkin type; indeed we found that male flower type is an other variable that contribute to the separation in different gene pools. The contribution of leaf morphology to cultivar identification has been largely debated (Fenaroli 1945) and in most cases considered very poor, but on a larger scale of samples it is possible that the leaf shape presents a variation that, although low, has a solid genetic base.

Conclusions

The results of the analyses carried out on 68 chestnut trees grown in different areas of Piedmont Region pointed out the presence of a great phenotypic and genotypic diversity. The microsatellite analysis proved to be a reliable and suitable technique for the DNA profiling of chestnut cultivars and was very helpful for detecting homonymous and synonymous varieties. Morphological traits were able to separate the 4 genepools found in the germplasm but few of them resulted effective in discriminating cultivars.

Evaluation of the genetic heritage and population structure is crucial for leading a conservation strategy and sustainable utilization of the natural resources (Lang and Huang 1999). Chestnut heritage is at risk of genetic erosion because many orchards are old and abandoned and plants of minor cultivars are being cut and replaced by others with better traits for the market. In the last years, the mentioned problem has sharply increased due to the introduction in Europe of *Dryocosmus kuriphilus* (Yasumatsu) from China, with the risks that Euro-Japanese hybrids, such as 'Bouche de Bétizac' which is resistant to the pest (Sartor et al. 2009), may replace the *C. sativa* cultivars in the areas of more intensive cultivation.

The chestnut cultivars described in this work represent an important and valuable source of biodiversity which should be protected and preserved. Germplasm collections play an essential role in this task; in this context the University of Torino established in 2005 a germplasm collection field of the chestnut genetic diversity ('Centro Regionale di Castanicoltura' located in Cuneo province, northwestern Italy) with the financial support of three public partners (Regione Piemonte, Ente Gestione Parchi e Riserve Cuneesi, and Comunità Montana delle Alpi del Mare).

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TABLES

Table 1 List of 68 *Castanea sativa* individuals sampled in this study, their cultivar name, number of accessions, tree code, geographic origin (Valley of cultivation) and prevalent fruit use. (P-SW: south-western Piedmont, P-W: western Piedmont, P-SE: south-eastern Piedmont).

Cultivar	N° of accessions	Tree code	Valley of cultivation	Prevalent fruit use
Borgna	1	CEVA07	Ceva (P-SE)	Drying, flour
Bracalla	1	MACC05	Maira (P-SW)	Fresh
Brunette	1	MACC08	Maira (P-SW)	Fresh
Ciapastra	2	TANA02, TANB02	Tanaro (P-SE)	Drying, flour
Ciaulina	1	CHIA02	Susa (P-W)	Fresh
Crou	1	PESA02	Pesio (P-SW)	Fresh
Frattona	2	CEVA01, CEVA03	Ceva (P-SE)	Drying, flour
Gabbiana	3	CEVA05, CEVA06	Ceva (P-SE)	Drying, flour
		TANE01	Tanaro (P-SE)	Drying, flour
Gaggia	1	TAND03	Tanaro (P-SE)	Drying, flour
Garrone Nero	5	GRAA04, GRAA06	Maira (P-SW)	Fresh
		PESC01, PESD01, PESE02	Pesio (P-SW)	Fresh
Garrone Rosso	5	GRAA01, STUB02	Maira (P-SW)	Fresh, marrons glacés
		PESD02, PESE01, PESF01	Pesio (P-SW)	Fresh, marrons glacés
Gentile	5	GRAC01	Maira (P-SW)	Fresh
		PESA03, PESD04, PESE03, PESF02	Pesio (P-SW)	Fresh
Gioviasca	2	PELA07, PELB03	Pellice (P-W)	Fresh
Madonna	3	MONA02, MONA03, MONA04	Roero (P-SW)	Fresh
Marrone di Chiusa Pesio	2	PESA01, PESB01	Pesio (P-SW)	Marrons glacés, fresh
Marrone di Luserna	1	PELC01	Pellice (P-W)	Marrons glacés, fresh
Marrone di Roccaverano	2	ROCB02, ROCB03	Roccaverano (P-SE)	Marrons glacés, fresh
Marrone di Val Susa	1	SUSB02	Susa (P-W)	Marrons glacés, fresh
Marrubia	1	PESF04	Pesio (P-SW)	Fresh, candying
Martiniana	1	TAND02	Tanaro (P-SE)	Drying, flour
Muraie	1	MACA01	Maira (P-SW)	Fresh
Neirana	2	PELA06	Pellice (P-W)	Fresh
		SUSF02	Susa (P-W)	Fresh
Pelosa	2	CHIA01	Susa (P-W)	Drying, flour
		PELC04	Pellice (P-W)	Drying, flour
Pelosa Piccola	1	PELB02	Pellice (P-W)	Drying, flour
Precoce di Brignola	1	PESG01	Pesio (P-SW)	Fresh
Primemura	1	CHIB01	Susa (P-W)	Fresh
Pugnenga	2	MACA03	Maira (P-SW)	Fresh
		PELA08	Pellice (P-W)	Fresh
Rian de Buire	1	TANB01	Tanaro (P-SE)	Drying, flour
Rubiera	3	MACC01, MACC03, MACC07	Maira (P-SW)	Fresh
Ruiana	1	PELA04	Pellice (P-W)	Fresh
Selvaschina	1	GRAB02	Maira (P-SW)	Fresh
Servai d'l'oca	1	MACB03	Maira (P-SW)	Fresh
Siria	2	GRAC02, MACC02	Maira (P-SW)	Drying, flour
Solenca	2	PELA03	Pellice (P-W)	Fresh
		SUSE01	Susa (P-W)	Fresh
Spinalunga	1	TANE02	Tanaro (P-SE)	Fresh
Tempuriva	4	PELD01	Pellice (P-W)	Fresh
		PESD03, PESF03	Pesio (P-SW)	Fresh
		STUA02	Maira (P-SW)	Fresh
Travisò	1	TAND01	Tanaro (P-SE)	Drying, flour

Table 2 Descriptors used for morphological traits of nuts, leaves and inflorescences of *Castanea sativa* accessions

Descriptors	Source	Trait description
Burs and nuts		
Nut: ripening time	UPOV 1989, Bounous et al. 2002	Very early: before 15 September Early: 15-30 September Medium: 1-15 October Late: 16-31 October Very late: after 1 November
Bur: density of spines	Bolvanský and Mendel 2001	Low Medium High
Bur: length of spines (mm)	Bolvanský and Mendel 2001	Short: until 7 mm Medium: 7,1-14,9 mm Long: 15-25 mm
Bur: number of filled nuts	Modified from Bolvanský and Mendel 2001	Number of filled nuts calculated on 25 fruits
Nut: size (number of nuts per kg)	Bounous et al. 2002	Very big < 60/kg Big: 61-80/kg Medium: 81-100/kg Small:101-120/kg Very small: >120/kg
Nut: colour detected according to a visual scale	UPOV 1989	Light brown Brown Dark brown Reddish brown Blackish brown
Nut: width/height ratio		
Nut: shape	Bounous et al. 2002	Conical Sub-conical Sub-spherical Ellipsoidal Sub-rectangular
Nut: hairiness		Absent Present: only around the torch Present: around the torch and downward Present: spread all over the nut
Nut: hilum length/width ratio	Modified from UPOV 1989	
Nut: percentage of double nuts or multiple-embryo nuts	Bounous et al. 2002	Null (o) Low (1-4) Moderate (5-8) High (8-12) Very high (>12)
Nut: pellicle adhesion to kernel	Bounous et al. 2002	Free (not adherent) Partially adherent Completely adherent
Nut: pellicle intrusion	Modified from UPOV 1989	Present, very prominent Present, but not much prominent Absent
Fully developed leaves		
Leaf: upper page aspect		Smooth Semi-rough Rough
Leaf: hairiness		Absent

		Present
Leaf: shape		Ovate-lanceolate Lanceolate
Leaf: petiole length (cm)	Bolvanský and Mendel 2001	
Leaf: length/width ratio of foliar blade	Modified from UPOV 1989	
Inflorescences		
Male flower type	Modified from UPOV 1989	Astaminate Brachistaminate Longistasminate Mesostaminate
Length of unisexual catkins (cm)	Modified from UPOV 1989	

Table 3 Polymorphism of 10 SSR loci for 36 chestnut genotypes. A: number of alleles, N_G= number of genotypes, H_E: expected heterozygosity, H_O: observed heterozygosity, N_A: Estimated frequency of null alleles, PI: probability of identity

LOCUS	**A**	**N_G**	**H_E**	**H_O**	**N_A**	**PI**
CsCAT1	8	15	0.774	0.861	-0.049	0.084
CsCAT3	13	20	0.807	0.722	0.047	0.056
CsCAT4	5	8	0.662	0.694	-0.019	0.166
CsCAT6	14	20	0.826	0.889	-0.034	0.052
CsCAT16	7	12	0.651	0.694	-0.026	0.157
CsCAT17	8	14	0.753	0.861	-0.061	0.096
EMCs15	4	9	0.618	0.639	-0.013	0.211
QpZAG110	7	12	0.736	0.694	0.024	0.115
QpZAG119	9	14	0.757	0.833	-0.044	0.095
QrZAG96	5	7	0.593	0.667	-0.046	0.213
Cumulative PI						2.96×10^{-10}

Table 4 Cultivar list redrawn on the basis of the genetic analysis (one cultivar = one unique genotype). Cases of homonymy are indicated with the same cultivar name followed by a different number; the original names (Table 1) of synonymous accessions are in Italic. In the last column the gene pool identified by Structure software is reported.

Cultivar	N° of accessions	Names used in table 1	Tree code	Structure gene pool (% inferred ancestry)
'Borgna'	1	Borgna	CEVA07	GREEN (97)
'Bracalla'	1	Bracalla	MACC05	YELLOW (87)
'Brunette'	1	Brunette	MACC08	RED (68)
'Ciapastra 1'	2	Ciapastra	TANB02	GREEN (74)
		Rian de Buire	TANB01	
'Ciapastra 2'	1	Ciapastra	TANA02	BLUE (48)
'**Frattona'**	2	Frattona	CEVA01	GREEN (89)
		Frattona	CEVA03	
'**Gabbiana 1'**	2	Gabbiana	CEVA06	GREEN (97)
		Gabbiana	TANE01	
'Gabbiana 2'	1	Gabbiana	CEVA05	YELLOW (56)
'Gaggia'	1	Gaggia	TAND03	GREEN (96)
'**Garrone Nero'**	5	Garrone Nero	GRAA04	RED (87)
		Garrone Nero	GRAA06	
		Garrone Nero	PESC01	
		Garrone Nero	PESD01	
		Garrone Nero	PESE02	
'**Garrone Rosso'**	6	Garrone Rosso	GRAA01	RED (74)
		Garrone Rosso	PESD02	
		Garrone Rosso	PESE01	
		Garrone Rosso	PESF01	
		Garrone Rosso	STUB02	
		Crou	PESA02	
'**Gentile'**	5	Gentile	GRAC01	RED (79)
		Gentile	PESA03	
		Gentile	PESD04	
		Gentile	PESE03	
		Gentile	PESF02	
'**Gioviasca'**	2	Gioviasca	PELA07	BLUE (95)
		Gioviasca	PELB03	
'**Madonna'**	3	Madonna	MONA02	YELLOW (88)
		Madonna	MONA03	
		Madonna	MONA04	
'**Marrone**'	7	*Marrone di Chiusa Pesio*	PESA01	RED (95)
		Marrone di Chiusa Pesio	PESB01	
		Marrone di Luserna	PELC01	
		Marrone di Roccaverano	ROCB02	
		Marrone di Roccaverano	ROCB03	
		Marrone di Val Susa	SUSB02	
		Marrubia	PESF04	
'Martiniana'	1	Martiniana	TAND02	GREEN (93)
'Muraie'	1	Muraie	MACA01	YELLOW (62)
'Neirana 1'	1	Neirana	PELA06	BLUE (73)
'Neirana 2'	1	Neirana	SUSF02	YELLOW (57)
'**Pelosa'**	3	Pelosa	CHIA01	YELLOW (89)
		Pelosa	PELC04	

		Ciaulina	CHIA02	
'Pelosa Piccola'	1	Pelosa Piccola	PELB02	BLUE (92)
'Precoce di Brignola'	1	Precoce di Brignola	PESG01	GREEN (53)
'Primemura'	1	Primemura	CHIB01	YELLOW (52)
'Pugnenga 1'	1	Pugnenga	MACA03	RED (61)
'Pugnenga 2'	1	Pugnenga	PELA08	BLUE (93)
'**Rubiera'**	3	Rubiera Rubiera Rubiera	MACC01 MACC03 MACC07	RED (79)
'Ruiana'	1	Ruiana	PELA04	BLUE (81)
'Selvaschina'	1	Selvaschina	GRAB02	RED (66)
'Servai d'l'oca'	1	Servai d'l'oca	MACB03	YELLOW (78)
'**Siria'**	2	Siria Siria	GRAC02 MACC02	GREEN (96)
'Solenca 1'	1	Solenca	SUSE01	RED (91)
'Solenca 2'	1	Solenca	PELA03	YELLOW (77)
'Spinalunga'	1	Spina Lunga	TANE02	GREEN (95)
'**Tempuriva 1'**	3	Tempuriva Tempuriva Tempuriva	PESD03 PESF03 STUA02	BLUE (84)
'Tempuriva 2'	1	Tempuriva	PELD01	BLUE (93)
'Travisò'	1	Travisò	TAND01	GREEN (96)

FIGURE CAPTIONS

Fig. 1 Analysis of population structure according to a Bayesian clustering method. The Piedmont chestnut population derive its genetic pool from 4 populations of inferred origin. The figure shows quantitative analysis of the genetic structure for the 36 genotypes. Each bar represents a single individual analyzed

Fig. 2 UPGMA dendrogram of 36 chestnut genotypes based on 10 SSR loci

Fig. 3 Discriminant analysis for diversity for morphological traits of chestnut accessions using the gene pool identified by Structure as classification criterium.

SUPPLEMENTARY MATERIAL CAPTIONS

Online Resource 1 Alleles and their frequency in the Piedmont germplasm at 10 SSR loci.
(Alleles typical of a single genotype for each locus are pointed out in bold)

Online Resource 2 Genetic profiles of 36 *Castanea sativa* genotypes analyzed at 10 SSR loci (allele size in base pairs)

Online Resource 3a Description of morphological traits of nuts observed in 36 *Castanea sativa* cultivated genotypes

Online Resource 3b Description of morphological traits of leaves and inflorescences observed in 36 *Castanea sativa* cultivated genotypes