Castanea sativa sweet chestnut ancient trees across Europe: sources of genetic diversity

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Abstract

Sweet chestnut ancient trees constitute a reserve of genetic variability, which can be considered a great resource for management programmes of forest species.
The genetic characterization of these trees can be crucial to improve our knowledge of the molecular basis of local adaptation, identify selective drivers and explain the contemporary genetic variation of tree species. In this study, we performed a comprehensive field survey to uncover the occurrence of large old sweet chestnut trees in South-Central Italy, Spain and Britain. Eighty-eight ancient chestnut trees were genotyped using nuclear microsatellites (SSRs) with the main goals of assessing their genetic identity and providing hypotheses on the origin of this germplasm. Our final objective is to contribute towards knowledge and valorisation of these large old trees of potential interest for both genetic improvement and conservation of European sweet chestnut.

Keywords: sweet chestnut, ancient trees, genetic variability, microsatellite markers

INTRODUCTION

Large old trees are extraordinary organisms. They represent not only an historical, landscape and environmental heritage of inestimable value, but they are also witness of environmental changes and human activities.

It is essential to find, preserve and study these extraordinary individuals not only for their aesthetic appeal or their well-known historical, cultural and social significance (Blicharska and Mikusiński, 2014), but also because they harbour valuable and distinct genetic diversity, which could testify advantageous productivity traits and unique abilities to cope with biotic and abiotic stresses (Lindenmayer and Laurance, 2017; Bräutigam et al., 2013; Beghè et al., 2013). Consequently, giant trees can be considered a great resource for forest species management programmes.

Castanea sativa Mill. is the only European species of the genus *Castanea*: it is widespread across Western Europe, with strongholds in the Mediterranean region. The current distribution of the species is the result of both natural colonization processes and human intervention (Mattioni et al., 2013). Genetic variation in sweet chestnut populations has been extensively investigated using molecular neutral markers, revealing a substantial genetic diversity across Europe. These studies have also shed light on the origin of the current distribution of European populations, which can be traced back to different glacial refugia during the Last Ice Age (Martín et al., 2012; Mattioni et al., 2017; Poljak et al., 2017).

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Additionally, SSRs have been employed to confirm the genetic identity of key cultivars in Europe (Pereira-Lorenzo et al., 2017; 2019) and also to determine the existence of grafted or agglomerated elements of different sweet chestnut genotypes within individual ancient trees (Jarman et al., 2019a; 2019b).

Furthermore, genic markers have demonstrated a signal of adaptation associated with drought tolerance and resistance to *Phytophthora cinnamomi* in chestnut populations from contrasting environmental conditions in Europe (Alcaide et al., 2019; 2020; Castellana et al., 2021).

Unfortunately, this genetic diversity has been severely affected by different biotic and abiotic factors that endanger both the conservation of these forest systems and their potential production.

In this scenario the study of genetic variability of large old chestnut trees, that may constitute a reservoir of unique genotypes, can be crucial to improve our knowledge on the molecular basis of local adaptation, identify selective drivers and preserve the genetic diversity of the species.

In this work using microsatellite markers, we genotyped 88 sweet chestnut giant trees coming from Britain, Spain and Italy. We evaluated the individual's genetic diversity and the genetic distance among the 88 trees, with the objective of contributing to the knowledge and valorisation of these large old trees, and to highlight germplasm sources of potential interest for both genetic improvement and conservation of European sweet chestnut.

MATERIALS AND METHODS

Plant material and DNA extraction

In total, 88 ancient sweet chestnut trees were identified and collected. The Italian chestnut trees were identified by accessing the catalogues of the National Ministry of Agricultural, Food and Forestry policies (D.M. 757 19/4/2019, https://www.politicheagricole.it). Samples from Britain were furnished by the University of Gloucestershire while samples from Spain were collected by the University of Cordoba and University of Santiago de Compostela.

We selected individuals with a circumference larger than 5 metres and with an estimated age greater than 300 years. Several discrete stools were sampled to test for clonality from the oldest and largest European chestnut monumental trees, as the tree from Tortworth (Britain, Gloucestershire) and the trees from Italy (Sicily) known as “Cento Cavalli” and “Nave”, estimated 3,000 and 4,000 years old (Lewington and Parker, 2002). Genomic DNA was extracted by grinding 50 mg of fresh leaf and buds tissues and using the DNeasy96 Plant Kit (Qiagen) according to the manufacturer’s protocol.

Microsatellite analysis

Ten nuclear microsatellite markers (nSSRs) (CsCAT1, CsCAT2, CsCAT3, CsCAT6, CsCAT14, CsAT16, EMCs15, EMCs25, EMCs38, QpZAG7) developed in *Castanea sativa* (Buck et al., 2003; Marinoni et al., 2003) and *Quercus petraea* (Steinkellner et al., 1997) were selected and subsequently screened to evaluate the amplification capacity. Two multiplex reactions were arranged based on the size of the amplification products, with forward primers labelled with the fluorescent dyes 6-FAM, NED, PET and VIC. PCR reactions were performed using the Type-it Microsatellite PCR Kit (QIAgen, Hilden, Germany) in 12.5 µL total volume containing 20 ng of genomic DNA on a GeneAmp 2700 Thermal Cycler (Applied Biosystems, Foster City, USA). Cycling parameters were set as follows: 5 min at 95°C, 30 cycles for 30 sec at 95 °C, 90 sec at 57 °C, 30 sec at 72 °C, and a final step of 30 min at 60 °C. Amplification products (0.1–1 µL) were added to 9.80 µL of formammide and 0.20 µL of size standard Genescan-500 LIZ to define allele sizes. SSR products were run on an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystem, Foster City, USA) for separation and sizing.

The alleles were scored using GeneMapper v4.0 software (Applied Biosystem, Foster City, USA).

Data analysis

The probability of null alleles (F*null)* for each of the 10 nSSR loci analyzed was tested using the software FreeNA (Chapuis and Estoup, 2007). GenoDive 3.0 Software (Meirmans and Tiederen, 2004) was used to identify clonal individuals. Only single, not clonal, genetic profiles were included in the subsequent genetic analysis. The genetic diversity was evaluated using the software GenAlex 6.5 (Peakall and Smouse, 2008), Darwin 6 (Perrier and Acquemoud-Collet, 2006).

A Bayesian clustering approach with STRUCTURE V.2.3.4 (Pritchard and Wen, 2009) was also performed, using the admixture model. The most likely number of K was calculated using STRUCTURE HARVESTER (Earl and von Holdt, 2012). CLUMPack software was used for the graphical representation of STRUCTURE results (Jakobsson and Rosenberg, 2007).

RESULTS AND DISCUSSION

Locus EMCs25, which showed a high frequency value of null alleles (0.278), Fst including null alleles (INA) 0.1535 and Fst excluding null alleles (ENA) 0.1162, was excluded from the subsequent analyses.

The genetic diversity indices calculated considering the different geographic origin showed similar variability in the different areas (Table 1).

These results are comparable with those observed in the natural populations close to the ancient trees (Mattioni et al., 2017; Martin et al., 2012; Jarman et al., 2019a).

Table 1. genetic diversity indices considering the three geographical areas

		Ne	I	Ho	He	uHe	F
Italy	**Mean**	5.01	1.78	0.72	0.75	0.75	0.08
	SE	0.81	0.19	0.10	0.05	0.05	0.11
Spain	**Mean**	4.29	1.55	0.60	0.71	0.72	0.15
	SE	0.69	0.17	0.07	0.06	0.06	0.08
Britain	**Mean**	4.89	1.76	0.70	0.74	0.75	0.07
	SE	0.88	0.17	0.06	0.04	0.04	0.06

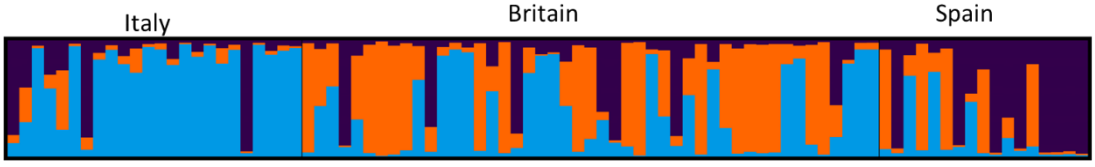

Figure . 1 Structure analysis of samples from Italy, Britain and Spain. Gene pool A (blue), gene pool B (purple), genepool C (orange)

Figure 1 presents the results of the STRUCTURE analysis of ancient sweet chestnut trees. The analysis revealed the presence of three main gene pools for K=3. The majority of Italian

samples are included in one gene pool (blue) while the majority of the Spanish samples are included in the second group (purple), with some samples belonging to the third pool (orange). Notably, the British samples showed evidence of admixture of the three groups, confirming the relationship between British chestnut samples and chestnut germplasm from Portugal, Spain, France and Italy described in previous studies (Jarman et al., 2019a).

The results of the UPGMA analysis based on the Nei genetic distance conducted on all the European accessions are reported in Figure 2. In green are indicated samples from Italy, in blue samples from Britain and in red samples from Spain. The oldest chestnut tree in Europe, known as "Centocavalli" and located in Sicily Italy, exhibited the highest genetic differentiation among all the assessed samples. Following this, a second tree located in Sicily, called "S. Alfio", displayed a similar pattern of genetic distinctiveness. It is noteworthy that an ancient tree sample from England is close to "Cento Cavalli" and both these samples are genetically close to the large old tree at Tortworth and a sample from Spain. No evident groups based on the geographic origin can be observed among the European samples.

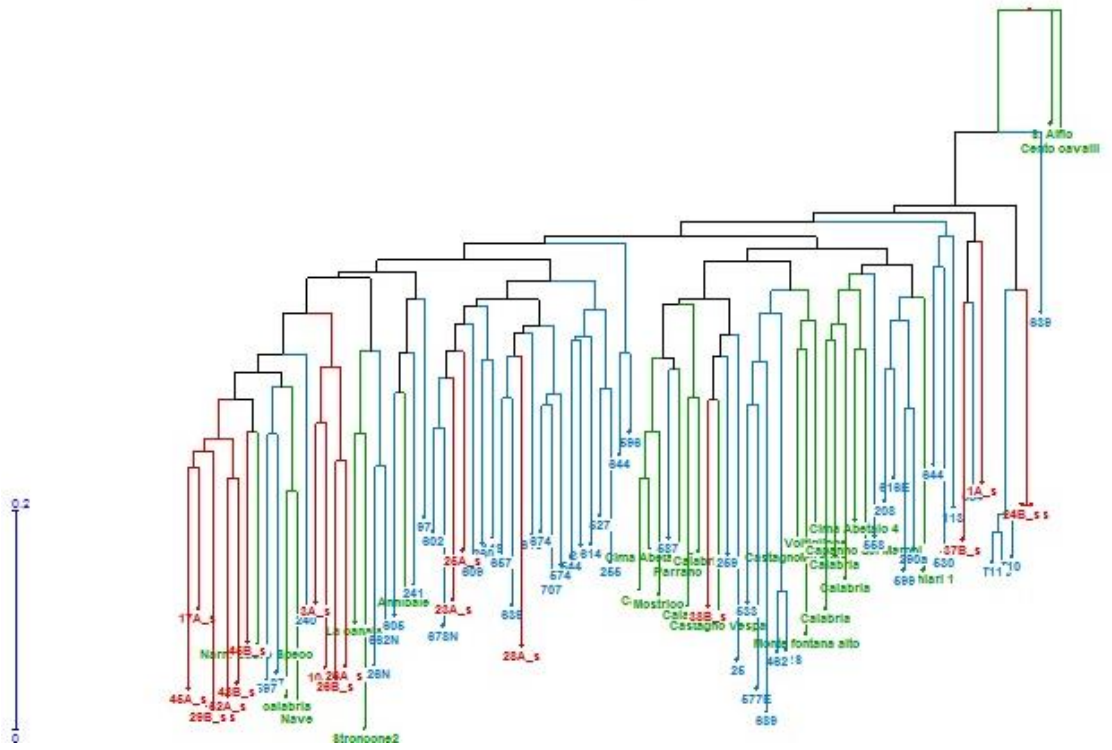

Figure 2. Dendrogram of sweet chestnut ancient samples from Italy (green) , Britain (blue) and Spain (red)

To study the genetic similarity among the Italian samples a UPGMA analysis based on Nei genetic distance was conducted (Figure 3). The different colours indicate different provenances: samples from Sicily in red, samples from Central Italy in green, from Northern Italy in blue and from Southern Italy in orange. Interestingly, the samples seem grouped with a geographic coherence. The oldest sample from Sicily, "Cento Cavalli", appears more divergent, and close to two other samples from Sicily ("S. Alfio" and "Monte Fontana"). Three other samples from Sicily ("Nave", "Monte Fontana" and "Vespa"), are close to samples from central Italy and a sample collected in a monastery in Umbria region. This representation of genetic similarity is probably owing to natural dispersion and human transport of plant material, as was identified in studies of British sweet chestnut trees (Jarman et al., 2019b).

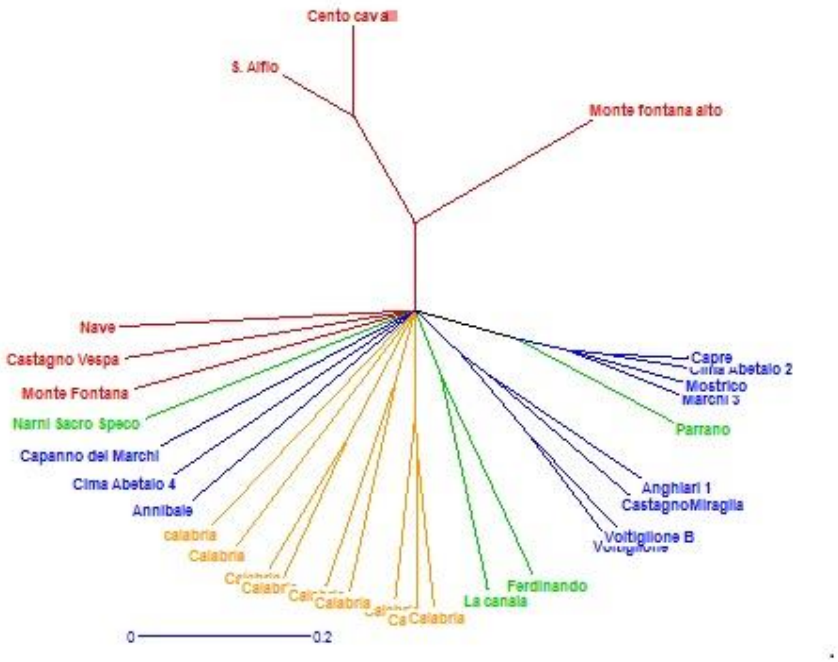

Figure 3. UPGMA analysis based on Nei genetic distance of Italian ancient chestnut trees. In red samples from Sicily, in orange sample from Calabria, in green from central Italy in Blue from northern-central Italy

Comparing genetic distance amongst all these European samples, the chestnut tree "Cento Cavalli" seems to be the most divergent. This tree is considered the most ancient tree in Europe and its genotype can be considered a relict of ancient gene pools.

CONCLUSIONS AND PERSPECTIVES

Until now, few studies have been carried out to evaluate the unique features of long life and pathogen resistance of the ancient chestnut trees (Topcu et al., 2009). The present work represents the first extensive study based on nuclear SSRs markers aiming to describe the individual genetic identity of European ancient sweet chestnut trees. We demonstrated that considerable genetic variability resides in the large old trees. The "Cento Cavalli", perhaps the oldest tree in Europe, showed the highest divergence confirming the individuality of its ancient genotype.

Undoubtedly, human efforts, particularly by mountain communities, have played a crucial role in maintaining these 'iconic' sweet chestnut trees. However, it cannot be discerned whether these plants survived because they were abandoned from cultivation, or because they possess favourable genetic characteristics that enable them to express distinct quality traits or enhanced resistance to environmental stresses that occurred in the past centuries. These old plants represent an important source of genetic variability that must be preserved

Future studies should focus on monitoring and sampling chestnut ancient trees all over the European range and applied recent genomic techniques to test adaptive potential and resilience of these extraordinary organisms to environmental changes.

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