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Genetic monitoring of traditional chestnut orchards reveals a complex genetic structure

M.A. Martín[1] · E. Monedero[2] · L.M. Martín[2]

Abstract

· *Key message* This study presents the results of a systematic genetic analysis between wild and cultivated chestnuts in an orchard in southern Spain, highlighting a complex structure and considerable genetic diversity and opening the possibility to generalize this approach to other Mediterranean orchards.

· *Context* Tree genetic monitoring offers a good opportunity to evaluate populations and preserve their long-term adaptive evolutionary potential. Chestnut is a multipurpose species of high economic importance in the Mediterranean basin and considered an example of integration between natural and man-driven distribution of diversity under changing environmental and historical conditions. Due to its multipurpose

Contribution of the co-authors M. Angela Martín participated in the design of the experiment, ran the data analysis, led the discussion and wrote the paper. Elvira Monedero participated in the collection of data in the field and conducted the laboratory analyses. Luis Miguel Martín designed the experiment, conducted the collection of data in the field, supervised the work and coordinated the research project.

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characteristics, man influenced its populations (grafting/sexual propagation) and a complex genetic structure is expected.

· *Aims* We monitored the trees of a chestnut orchard for studying the genetic diversity and relationship in grafts and rootstocks and detecting possible response in its adaptive potential.

· *Methods* For this, morphological traits and genomic and genic microsatellite markers were used.

· *Results* Chestnut trees showed considerable genetic structure, with high level of clonality in the varieties and genetic diversity in rootstocks. The similarity analysis revealed a different clustering pattern for varieties, detecting higher variability for genomic microsatellite markers. Rootstocks harboured a high level of diversity, not previously described, and not contained in the genetic information from populations and varieties from the same region.

· *Conclusion* Results contribute to understanding the human role in the management of chestnut and demonstrate that rootstocks constitute an unexploited reservoir of variation valuable for conservation strategies against stress factors and future and unpredictable environmental changes.

Keywords *Castanea sativa* · Genetic resources · *On farm* conservation · Clonality · Rootstocks

1 Introduction

Genetic monitoring offers a good opportunity for evaluating the status of tree population genetic resources over time, preserving their long-term adaptive evolutionary potential and detecting possible critical signals that demand management action (Schwatz et al. 2006; Graudal et al. 2014). This implies a systematic survey of the amount of genetic variation, genetic composition and spatial genetic structure to detect potential

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changes in these parameters that may result in loss of gene-level variability (Laikre et al. 2008).

Tree genetic resources exist at different levels of domestication, and the landscapes within which they are located are themselves domesticated to a greater or lesser extent (Michon 2005). In this respect, traditional agroecosystems involve the integration of trees with crops and livestock production and exhibit common features such as a high diversity of species, the use of diversified traditional varieties and low inputs associated with traditional farming practises (Garrity 2004). In these systems, *on farm* conservation is practised to preserve landraces in areas in which they were originally cultivated and with traditional technologies that have been practised by farmers for millennia (Maxted et al. 1997). Nevertheless, this conservation will only be effective if it is possible to assess the genetic diversity conserved in these agroecosystems and how they can evolve (Graudal et al. 2014).

The Mediterranean basin still harbours traditional agroecosystems of particular importance for preserving biodiversity, and sweet chestnut (*Castanea sativa* Miller) is a good example. This multipurpose species of high economic importance is valued not only for fruit and timber production but also for its contribution to the landscape and environment. It is accepted that domestication events in chestnut started several millennia ago and were characterized by clonal propagation and selection of the best genotypes that resulted in a population structure far from what would be expected in a purely natural situation. Thus, chestnut genetic structure is complex and depends on the type of management: orchards (dedicated to fruit production), coppices (dedicated to timber production) and naturalized populations (Grossmann and Romane 2004). In particular, orchards are constituted by trees of advanced and heterogeneous age, and chestnut cultivation involves grafting of traditional cultivars onto rootstocks. Thus, the trees from a given variety are clones, whereas rootstocks are the result of the germination of nuts selected by growers for its superior traits from different chestnut varieties or from wild populations (Martín et al. 2009; Marinoni et al. 2013). Furthermore, trees can show one or more varieties in the aerial part and branches without grafts (Martín et al. 2007).

These traditional farming systems are common throughout the Mediterranean region and constitute important elements of production systems and the farmers' livelihood strategies. The majority of this germplasm is rich in excellent cultivars resulting from the selection for specific nut traits by farmers. Thus, the landraces are highly adapted to the local environment and are likely to contain locally adapted alleles of gene complexes (Martín et al. 2016). In this respect, molecular studies indicate the existence of several local domestication events in the species supported by the association

between chestnut cultivars and their origin areas and also by the high diversity of different clonally propagated cultivars found in all countries where chestnut is traditionally grown (Gobbin et al. 2007; Martín et al. 2009, 2010; Pereira-Lorenzo et al. 2010).

Nevertheless, in the last decades, there has been a strong decline in chestnut cultivation closely associated with ink disease caused by *Phytophthora cinnamomi* Rands. This oomycete infects the root system, causing the wilting and death of chestnut trees (Crandall et al. 1945). Its impact on the production has dramatic consequences in local economies that discourage farmers to continue growing chestnut. In some parts in Europe, the impact of *P. cinnamomi* has been mitigated by the use of rootstocks that come from the hybridization between *C. sativa* and two Asian tolerant species (*Castanea crenata* and *Castanea mollissima*) (Fernández-López et al. 2001). However, these hybrids displayed some problems as graft incompatibility reactions between rootstock and cultivar, different agronomic traits not accepted by growers and/or consumers and poor adaptation to climatic conditions. In particular, in southern Europe, the use of these hybrids has proved to be unsuitable due to difficulties of adaptation of this resistant material to southern latitudes. For this reason, local rootstocks from *C. sativa*, developed by growers in different edaphoclimatic regions from central and southern Spain, are usually the ones which are best adapted to the warmer and drier local conditions and to the traditional varieties (Pereira-Lorenzo et al. 2010; Dinis et al. 2011).

Genetic variation in orchards and naturalized populations has been evaluated separately (Martín et al. 2012; Mattioni et al. 2013); thus, there are no genetic data on their possible relationship. Furthermore, the role of rootstocks in this genetic diversity has not been addressed in any study. It has been speculated that the genetic structure contained in rootstocks could be similar to that contained in the varieties or in the populations although there are no empirical data that support this hypothesis.

Molecular markers provide valuable information about the genetic status and biological processes of populations, making genetic monitoring increasingly feasible and cost-effective (Allendorf et al. 2010). Microsatellite markers (simple sequence repeats (SSRs)) have become the most used markers for studying forest genetics, because they are highly polymorphic, codominant and widespread across the genome (Glaubitz and Moran 2000). However, there has been renewed interest in complementing the analysis of neutral markers with assessment of loci that may be directly involved in responses to processes such as environmental changes (Hoffman and Willi 2008). The increased availability of DNA sequences has permitted the development of expressed sequence tag (EST)-based SSR markers from EST sequences expressed in different physiologic conditions of plants. Their main advantage compared with genomic SSRs is that they are present in

expressed regions of the genome, thus potentially having known functions (Varshney et al. 2005).

The aim of this study was the genetic monitoring of the productive system of traditional chestnut orchards. For this purpose, an orchard was selected in which previous observations suggested the existence of genetic diversity and the use of grafting. It is important to note that the situation of the orchard described in the study is an archetype of those chestnuts dedicated to fruit production in the Mediterranean basin. The specific objectives were to (1) study the current status of the genetic diversity harboured in the orchard, in the aerial part and rootstocks; (2) confirm the existence of clonal varieties; (3) test the potential of EST-SSR markers to detect possible response in the adaptive potential of chestnut varieties and conduct genetic monitoring; (4) study the genetic relationship between clonal varieties vs. rootstocks; and (5) compare the genetic diversity obtained with that for the rest of Andalusia.

2 Material and methods

2.1 Plant material

The study was conducted in the farm field 'La Chaparra', located in the Natural Park Sierra Norte de Sevilla (Andalusia, southern Spain). This orchard covers an area of 1.19 ha and has a density of 35 trees/ha (Fig. 1). For the analysis, 25 large and vigorous trees were catalogued and data of girth of rootstocks and the type of male catkins were recorded (Table 1).

Chestnut cultivation for fruit production is based on a set of traditional varieties grafted onto seedling rootstocks. Thus, trees can present two or more genotypes: (i) the rootstock part that comes from sexual reproduction and (ii) the productive part that comes from vegetative multiplication. Thus, the set of rootstocks may be comparable with a natural population (considering that this traditional orchard do not use hybrids as rootstocks) and the varieties with clones of identical genotypes.

To determine whether trees were grafted, and considering the information above, different samples of leaves were collected for DNA extraction and posterior molecular analysis. Thus, for each tree, one sample was taken from the aerial part and another from the rootstock. Furthermore, in those cases in which trees were suspected to be grafted with more than one variety, two different leaves were collected from the aerial part (named with A and B, Table 1).

2.2 Morphological and molecular characterization

According to flowering, four different types of male catkins are described by UPOV (1989): longistaminate, mesostaminate, braquistaminate and astaminate. These
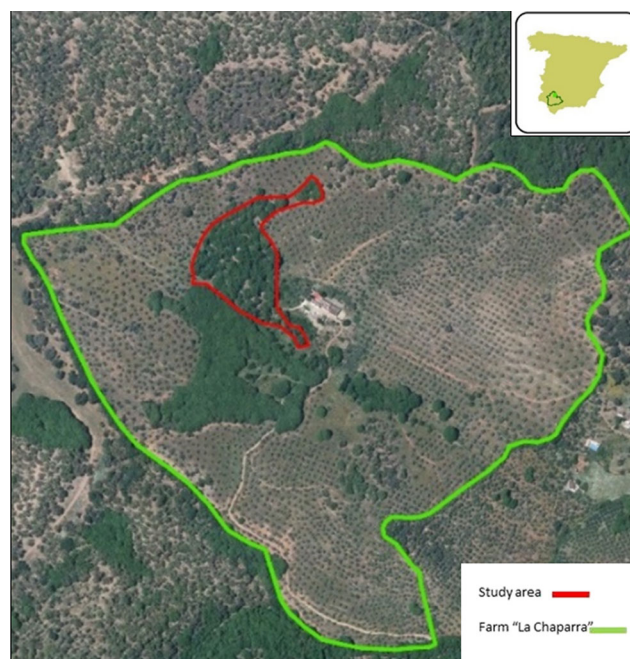

Fig. 1 Location of the farm field where the study has been conducted

inflorescences produce different amount of pollen, from longistaminate type that generate large amounts of pollen to astaminate type where pollen is absent.

The type of male catkin served as first indicator to detect the possible existence of more than one variety in the same tree. In the study, two different situations were found: (i) trees that showed only one type of male catkin and (ii) trees with two types of male catkins. In this case, two different samples of leaves were taken that were distinguished with A/B (Table 1).

For molecular analysis, DNA was extracted from 20 mg of lyophilized leaves according to DNeasy Plant Mini Kit protocol (Qiagen, CA). A set of eight neutral microsatellites (*CsCAT1*, *CsCAT2*, *CsCAT3*, *CsCAT6*, *CsCAT14*, *CsCAT16*, *EMCs25* and *EMCs38*) developed in *C. sativa* (Marinoni et al. 2003; Buck et al. 2003) was tested. Furthermore, nine functional microsatellite primer pairs (*FIR030*, *GOT014*, *PIE227*, *PIE228*, *PIE233*, *PIE260*, *POR009*, *POR026* and *WAG004*) were used. These primers were selected from EST expressed in bud tissue of *Quercus robur* and *Quercus petraea* (EVOLTREE database http://www.evoltree.soton.ac.uk/portal). The EST-SSRs were previously mapped on a F_1 intraspecific cross (*C. sativa* × *C. sativa*) and interspecific cross (*Q. robur* × *Q. petraea*), and each locus belongs to a different linkage group (Durand et al. 2010).

PCR reaction mixture consisted of 12.5 μL total volume containing 20 ng of genomic DNA following the Qiagen Type-it protocol. Cycling parameters were 15 min at 95 °C; 30 cycles of 30 s at 94 °C, 90 s at 60 °C and 1 min at 72 °C; and a final step of 30 min at 72 °C. Amplification products (0.1–1 μL) were added to 20 μL formamide and 0.3 μL

Table 1 Chestnut accessions analysed in which it is indicated: part of the tree where the sample was taken, type of male catkin, graft detection and girth of rootstock

Accession	Part of the tree	Male catkin	Graft	Girth (cm)
SE-01-1.1	Rootstock			9.20
SE-01-1.1var	Graft	Longistaminate	Yes	
SE-01-1.2	Rootstock			10.50
SE-01-1.2var	Graft	Astaminate	Yes	
SE-01-1.3	Rootstock			5.00
SE-01-1.3Avar[a]	Rootstock	Longistaminate	No	
SE-01-1.3Bvar[a]	Graft	Astaminate	Yes	
SE-01-1.4	Rootstock			7.00
SE-01-1.4Avar[a]	Rootstock	Longistaminate	No	
SE-01-1.4Bvar[a]	Graft	Astaminate	Yes	
SE-01-1.5	Rootstock			4.90
SE-01-1.5Avar[a]	Graft	Astaminate	Yes	
SE-01-1.5Bvar[a]	Rootstock	Longistaminate	No	
SE-01-1.6	Rootstock			3.00
SE-01-1.6var	Graft	Astaminate	Yes	
SE-01-1.7	Rootstock			5.10
SE-01-1.7var	Graft	Astaminate	Yes	
SE-01-1.8	Rootstock			2.80
SE-01-1.8var	Graft	Astaminate	Yes	
SE-01-1.10	Rootstock			6.50
SE-01-1.10var	Graft	Astaminate	Yes	
SE-01-1.11	Rootstock			2.40
SE-01-1.11var	Graft	Astaminate	Yes	
SE-01-1.12	Rootstock			2.00
SE-01-1.12var	Graft	Astaminate	Yes	
SE-01-1.16	Rootstock			2.10
SE-01-1.16var	Rootstock	Mesostaminate	No	
SE-01-1.17	Rootstock			3.20
SE-01-1.17var	Rootstock	Longistaminate	No	
SE-01-1.18	Rootstock			2.15
SE-01-1.18var	Graft	Astaminate	Yes	
SE-01-1.19	Rootstock			5.50
SE-01-1.19var	Graft	Astaminate	Yes	
SE-01-1.20	Rootstock			5.40
SE-01-1.20var	Graft	Astaminate	Yes	
SE-01-1.21	Rootstock			2.40
SE-01-1.21var	Graft	Astaminate	Yes	
SE-01-1.22	Rootstock			2.10
SE-01-1.22var	Graft	Mesostaminate	Yes	
SE-01-1.23	Rootstock			9.90
SE-01-1.23var	Graft	Astaminate	Yes	
SE-01-1.24	Rootstock			4.90
SE-01-1.24var	Graft	Astaminate	Yes	
SE-01-1.25	Rootstock			2.80
SE-01-1.25var	Graft	Astaminate	Yes	
SE-01-1.27	Rootstock			2.60
SE-01-1.27var	Graft	Astaminate	Yes	
SE-01-1.28	Rootstock			2.50
SE-01-1.28var	Graft	Astaminate	Yes	
SE-01-1.29	Rootstock			2.70
SE-01-1.29var	Graft	Astaminate	Yes	
SE-01-1.30	Rootstock			6.50
SE-01-1.30var	Graft	Astaminate	Yes	

[a] Trees in which two samples were taken in canopy because there was doubt if they had two different grafts

Genescan-500 ROX and denatured at 95 °C for 5 min and run on an ABI PRISM 3100 DNA sequencer. Allele scoring was performed using the GeneScan 3.5 and Genotyper 3.7 softwares (Applied Biosystems).

2.3 Statistical analysis

To address genetic monitoring, we selected the operational indicator 'trends in population condition' proposed by Graudal et al. (2014) and the verifiable indicators related to the diversity in adaptive traits and population genetic structure. Nevertheless, it must be noted that we conducted an approximation to genetic monitoring considering that chestnut orchards are not natural populations. In this respect, genetic diversity parameters such as the number of alleles per locus (Na), the observed heterozygosity (Ho), the expected heterozygosity (He) and the number of private alleles (specific alleles detected in a concrete variety) were estimated using GENALEX 6 (Peakall and Smouse 2005). The inbreeding coefficient F_{IS} (Weir and Cockerham 1984) was calculated using ARLEQUIN 3.1 software (Schneider et al. 2000) and its deviation from zero tested by 10,000 allele permutations. Differentiation between varieties and rootstocks was calculated by F_{ST} according to Weir and Cockerham (1984) and its analogue, R_{ST}, according to Slatkin (1995). Furthermore, analysis of molecular variance (AMOVA) was conducted to quantify the proportion of genetic variation due to differences among rootstocks vs. varieties using ARLEQUIN 3.1 software (Schneider et al. 2000). Significance was tested using the nonparametric approach described in Excoffier et al. (1992) with 1000 permutations.

NTSYS 2.1 software (Exeter Software, Setauker, NY, USA) was used to (i) identify different varietal genotypes for the whole set of SSRs and EST-SSRs and to (ii) detect possible relationships between varieties vs. rootstocks. The band similarity coefficient of Lynch (1990) was calculated for paired comparison of all samples. This was tested by cophenetic matrix correlation during the reconstruction of a cophenetic matrix based on tree matrix (Rohlf and Fisher 1986).

The possible introgression between rootstocks and varieties was assessed using a Bayesian approach with STRUCTURE v.2.3.4 software (Pritchard et al. 2000). This attempts to reveal the genetic structure by placing individuals in K number of clusters. STRUCTURE was run using the admixture model on the whole dataset and the correlated allele frequencies (Falush et al. 2007; Hubisz et al. 2009). Based on the initial results, six independent runs (from 1 to 6) were performed for each K value, with a burn-in period of 10,000 steps followed by 10^5 MCMC replicates. To identify the number of clusters (K) that best explained the data, the rate of change on L(K) (ΔK) between successive K values was calculated according to Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012). The six runs for each simulation were averaged using CLUMPP software (Jakobsson and Rosenberg 2007) and represented graphically with DISTRUCT (Rosenberg 2004).

Finally, varietal information obtained in this study was compared with the Andalusian catalogue of traditional chestnut varieties. In this region, the main orchards dedicated to nut

production appear in Huelva and Malaga provinces and represent approximately the 70% of the species surface in the region. For the analysis, 12 genotypes previously identified in Huelva and 19 in Malaga (Martín et al. 2009) were selected and STRUCTURE v.2.3.4 software (Pritchard et al. 2000) was used. In this case, the software was run with the option of including prior information on the spatial location of varieties and using the admixture model on the whole dataset and the correlated allele frequencies (Falush et al. 2007; Hubisz et al. 2009).

3 Results

3.1 Graft detection

A total of 22 individuals displayed only one type of male catkin in the aerial part, and within them, molecular markers indicated that 20 were grafted (Table 1). There was a strong relationship between grafted chestnut and the type of male catkin, with more than 80% of them showing astaminate catkins (i.e. those that do not produce pollen). Furthermore, the two individuals that results showed were not grafted (SE-01-1.16var and SE-01-1.17var) displayed mesostaminate and longistaminate catkins (i.e. those that produce great amounts of pollen).

Three individuals with two different types of male catkin were detected (SE-01-1.3var, SE-01-1.4var and SE-01-1.5var). Results showed that all three trees were grafted, and samples A and B were different, and one of them had the same microsatellite profile as the basal part. This result can be explained by the fact that one aerial sample corresponded to the graft, while the other sample was from a branch of the rootstock.

3.2 SSR and EST-SSR-based varietal identification

After cataloguing grafted trees, we evaluated the varieties. The identification and genetic relationships among the varieties were depicted by two neighbour-joining dendrograms according to the two types of markers (Fig. 2).

The similarity analysis conducted using genomic SSRs detected nine genotypes among the 23 individuals analysed (Fig. 2a). Moreover, the level of similarity between groups of varieties was 0.18, and the cophenetic value was high and significant ($r = 0.953$; $P < 0.001$), indicating a good fit for the results obtained (Fig. 2a). The dendrogram showed two dominant genotypes shared by nine and seven individuals, respectively, whereas the remaining seven trees showed their own different genotype (Fig. 2a). Furthermore, there were two individuals (SE-01-1.23var and SE-01-1.29var) with a high degree of genetic similarity to the genotype shared by seven other trees.

A different grouping pattern was found with functional markers (Fig. 2b). In this case, EST-SSR loci displayed a lower level of discrimination, detecting only four genotypes in contrast to the nine found with SSRs, at a similarity level of 0.48 ($r = 0.969$; $P < 0.001$). In this case, the dendrogram also showed a clear separation between two principal genotypes with a high level of similarity (0.75) and with two individuals (SE-01-1.1var and SE-01-1.22var) of different genotypes, clustered further from the rest. It should be noted that the dendrogram discriminated individuals according to the type of male catkin, showing two similar groups characterized by the presence of astaminate catkins and two differentiated genotypes corresponding to mesostaminate and longistaminate catkins (Table 1; Fig. 2b).

The presence of exclusive alleles was analysed considering the four genotypes found using EST-SSRs. Thus, one private allele in locus *FIR030* was detected for the accession SE-01-1.1var corresponding to longistaminate and four private alleles (*PIE227*, *PIE228*, *POR09* and *WAG004*) for the accession SE-01-1.22var corresponding to mesostaminate catkins. Moreover, for the two dominant genotypes with astaminate catkins, four private alleles (*PIE227*, *PIE228*, *POR09* and *WAG004*) were found in the first group (represented by nine trees) and three alleles (*PIE233*, *GOT014* and *WAG004*) in the second group.

3.3 Genetic diversity in SSRs and EST-SSRs

Both genomic SSR and EST-SSR markers were polymorphic in the material evaluated with 53 and 36 alleles detected, respectively (Table 2). Allelic variation was in the range of 4–11 alleles in the genomic SSRs and 3–5 in EST-SSRs, with means of 6.6 and 4.0 alleles, respectively (Table 2). The level of gene diversity was higher for genomic SSRs than EST-SSRs (0.61 vs. 0.52), although this difference was not significant (Table 2). The inbreeding coefficient (F_{IS}), which is the measure of heterozygote deficit, showed significant deviation from zero in five loci, two in genomic SSR (*CsCAT2* and *EMCs25*) and three in EST-SSR markers (*FIR030*, *GOT014* and *PIE260*) (Table 2).

The polymorphism obtained in genomic SSRs within the rootstock population was higher than that obtained in varieties, with significant differences in the number of alleles and expected diversity (49 and 0.72 vs. 39 and 0.50, respectively). The same results were obtained for EST-SSRs, where rootstocks displayed higher levels of genetic diversity, although these differences were less pronounced (33 and 0.59 vs. 27 and 0.45). The values of F_{IS} were negative in rootstocks both for SSRs and EST-SSRs, and there were no cases in which this value deviated significantly from zero.

The coefficient of differentiation R_{ST} showed higher values than F_{ST} coefficient for both types of markers, although this
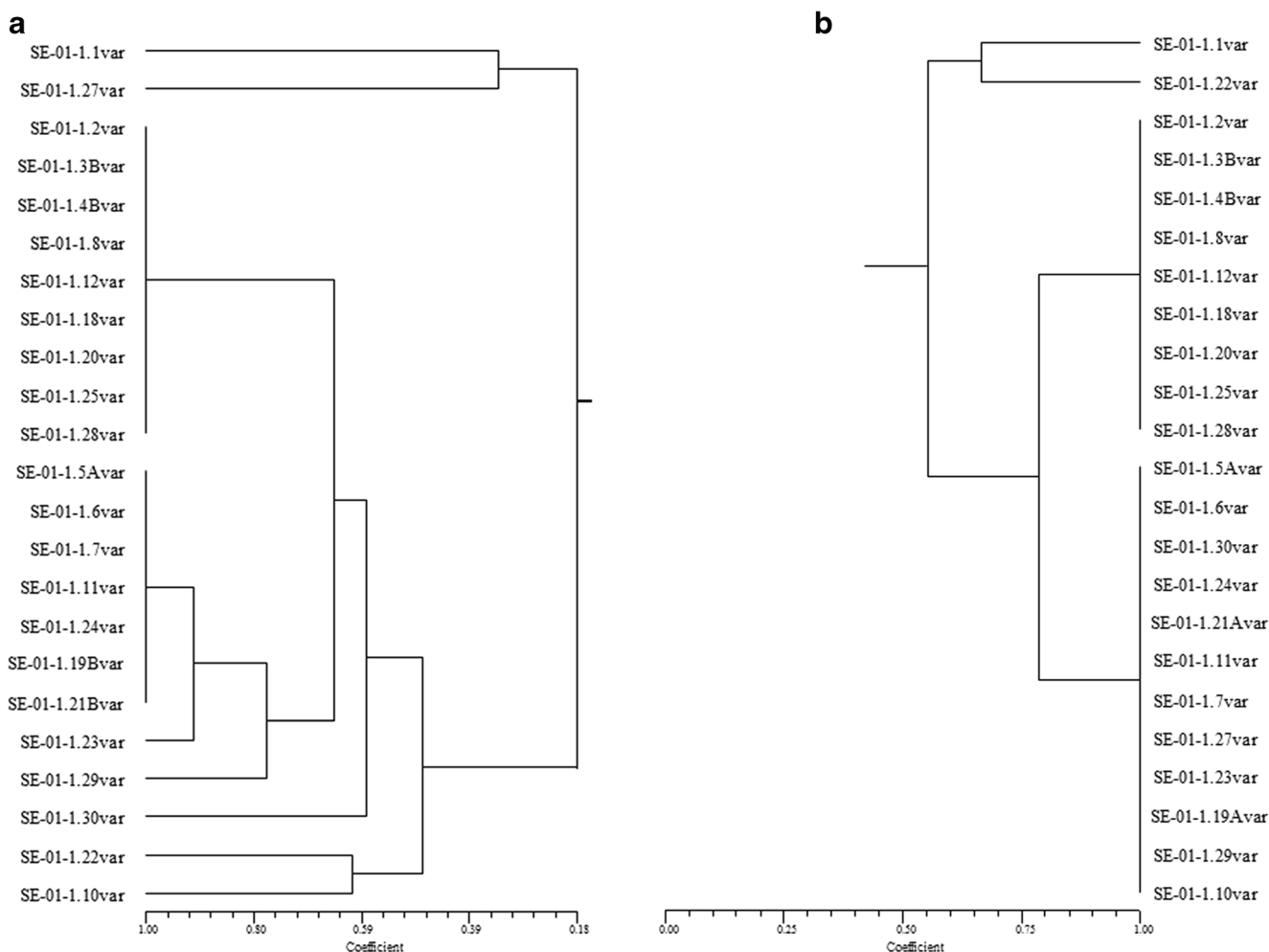
Fig. 2 Dendrogram showing the clustering patterns of chestnut traditional varieties using **a** SSR markers and **b** EST-SSR markers

difference was higher in the case of SSRs (0.25 vs. 0.14 for SSRs and 0.17 vs. 0.11 for EST-SSRs) (Table 2).

3.4 Genetic structure between rootstocks and varieties

To study the possible genetic relationship and structure between rootstocks and varieties, a similarity analysis was performed with a new cluster analysis using SSR markers (Fig. 3). The dendrogram distinguished two different groups at a similarity level of 0.30 ($r = 0.953$; $P < 0.001$), generally corresponding to rootstocks and varieties. Thus, one group included all rootstocks except accessions SE-01-1.8 and SE-01-1.16 and the other varieties except SE-01-1.1var, SE-01-1.22var and SE-01-1.27var (Fig. 3). Likewise, AMOVA analysis detected significant genetic differentiation between rootstocks vs. varieties in both types of markers, being this difference more pronounced in the case of neutral markers (22.01 vs. 17.98%, respectively) (Table 3). These results were corroborated by analysis of differences in the presence of private alleles: Rootstocks showed 23 exclusive

alleles not found in varieties (14 for SSRs and 9 for EST-SSRs), and varieties displayed 7 alleles not found in rootstocks (4 for SSRs and 3 for EST-SSRs) (Table 2). The results obtained with STRUCTURE were congruent with the clustering pattern obtained with the dendrogram, showing the most probable division at $K = 2$ that corresponded to rootstock and variety groups (Online Resource 1). Furthermore, possible introgression between rootstocks and varieties was detected in only five individuals (four rootstocks and one variety). In the case of rootstocks, the admixture value was between $0.4232 \leq q \leq 0.6202$, and for the variety, this value was $q = 0.3743$.

The genetic information found in the evaluated material, both in rootstocks and varieties, was compared with that reported in other studies in chestnut natural populations and traditional varieties from the same region (Andalusia), using Bayesian analysis with STRUCTURE. In the case of varieties, we assumed from previous results that varieties from Malaga and Huelva provinces have their own genetic integrity and were adapted to

Table 2 Genetic diversity found in rootstocks and varieties for the eight SSR loci and nine EST-SSRs

	Rootstocks					Varieties					All					
Locus	Na	Pa	Ho	He	F_{IS}	Na	Pa	Ho	He	F_{IS}	Na	Ho	He	F_{IS}	F_{ST}	R_{ST}
SSRs																
CsCAT1	5	1	0.76	0.70	−0.08	4	0	0.91	0.58	−0.59	5	0.84	0.64	−0.31		
CsCAT2	11	5	0.60	0.83	0.28	6	0	0.09	0.31	0.72	11	0.34	0.57	0.40*		
CsCAT3	2	0	1.00	0.50	−1.00	6	4	0.96	0.76	−0.25	6	0.98	0.63	−0.55		
CsCAT6	8	1	0.80	0.84	0.05	7	0	0.61	0.56	−0.09	8	0.70	0.70	0.00		
CsCAT14	4	0	0.72	0.70	−0.02	4	0	0.13	0.30	0.57	4	0.43	0.50	0.16		
CsCAT16	4	1	0.60	0.55	−0.09	3	0	0.96	0.60	−0.59	4	0.78	0.58	−0.35		
EMCs25	6	2	0.24	0.77	0.68	4	0	0.09	0.24	0.63	6	0.16	0.50	0.67*		
EMCs38	9	4	0.80	0.84	0.05	5	0	0.52	0.67	0.22	9	0.66	0.76	0.13		
Overall	49	14	0.69	0.72	−0.01	39	4	0.52	0.50	0.08	53	0.61	0.60	0.01	0.14	0.25
EST-SRs																
FIR030	3	1	0.52	0.65	0.19	3	1	0.08	0.15	0.47	4	0.30	0.53	0.43*		
GOT014	4	1	0.40	0.71	0.93*	3	0	0.04	0.58	0.93	4	0.04	0.73	0.95*		
PIE227	5	1	0.76	0.70	−0.08	4	0	0.96	0.57	−0.68	5	0.86	0.72	−0.20		
PIE228	5	3	0.68	0.67	−0.02	2	0	0.00	0.08	1.00	5	0.34	0.45	0.24		
PIE233	3	0	0.52	0.41	−0.26	3	0	0.88	0.63	−0.39	3	0.70	0.61	−0.16		
PIE260	3	1	0.68	0.59	−0.15	2	0	0.08	0.50	0.84	3	0.38	0.58	0.35*		
POR009	4	1	0.68	0.63	−0.09	3	0	0.92	0.51	−0.79	4	0.80	0.62	−0.30		
POR026	3	1	0.28	0.54	0.48*	2	0	0.84	0.50	−0.69	3	0.56	0.53	−0.05		
WAG004	3	0	0.48	0.40	−0.19	5	2	0.96	0.56	−0.73	5	0.72	0.51	−0.42		
Overall	33	9	0.52	0.59	−0.09	27	3	0.53	0.45	0.00	36	0.52	0.52	−0.01	0.11	0.17

A number of alleles per locus, *Pa* private alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, F_{IS} inbreeding coefficient, F_{ST} differentiation among populations according to Weir and Cockerham (1984), R_{ST} differentiation among populations according to Slatkin (1995)

*P<0.05

local conditions of the areas in which they were grown (Martín et al. 2007, 2009). In this respect, each province was considered a population containing the different genotypes identified therein. Thus, the Huelva population was consisted of 12 genotypes corresponding to 12 clonal varieties, Malaga of 19 and Sevilla of 9 (Online Resource 2). The results indicated that the most probable division with the strongest support in terms of log-likelihood values was for *K* = 2. This level of structure separated the samples into two groups: The first group (green cluster) comprised mainly varieties from Sevilla and Malaga and the second group (red cluster) varieties from Huelva (Fig. 4a).

In the comparison of populations and rootstocks, the most probable division was at *K* = 3, whereas for *K* > 3, the results were not consistent and membership analyses were unstable among runs. For *K* = 3, three clusters were identified with limited admixture among clusters (Fig. 4b). The first group comprised populations from southeastern Spain (SP01, SP05 and SP09, blue cluster); the second was made by populations from southwestern Spain (SP04, SP11 and SP14, yellow cluster), and the third group was only formed by samples from rootstocks (cluster red). Furthermore, only population SP12

displayed a high degree of admixture of clusters blue and yellow (Fig. 4b).

4 Discussion

This study presents the results of a systematic genetic structure analysis of chestnut traditional varieties, rootstocks and populations in an orchard in southern Spain. This material was surveyed using neutral and functional microsatellite markers with the main goal of enhancing our knowledge of cultivar identity, genetic variability and phylogenetic relationships.

The results indicate that despite being a region where chestnut is mostly used for timber production, the traditional fruit production system is based in clonal varieties, considering that only 2 of 25 individuals were not grafted. In this respect, nine varieties of clonal nature were identified using neutral microsatellite markers, with two of them considered dominant. It is possible that these results can be extrapolated to information provided by farmers in the area, concerning the existence of two varietal types, called 'Fina' and 'Boronda' (Gallardo 2002), but confirming this hypothesis will require additional studies. Furthermore, there were two individuals with a high
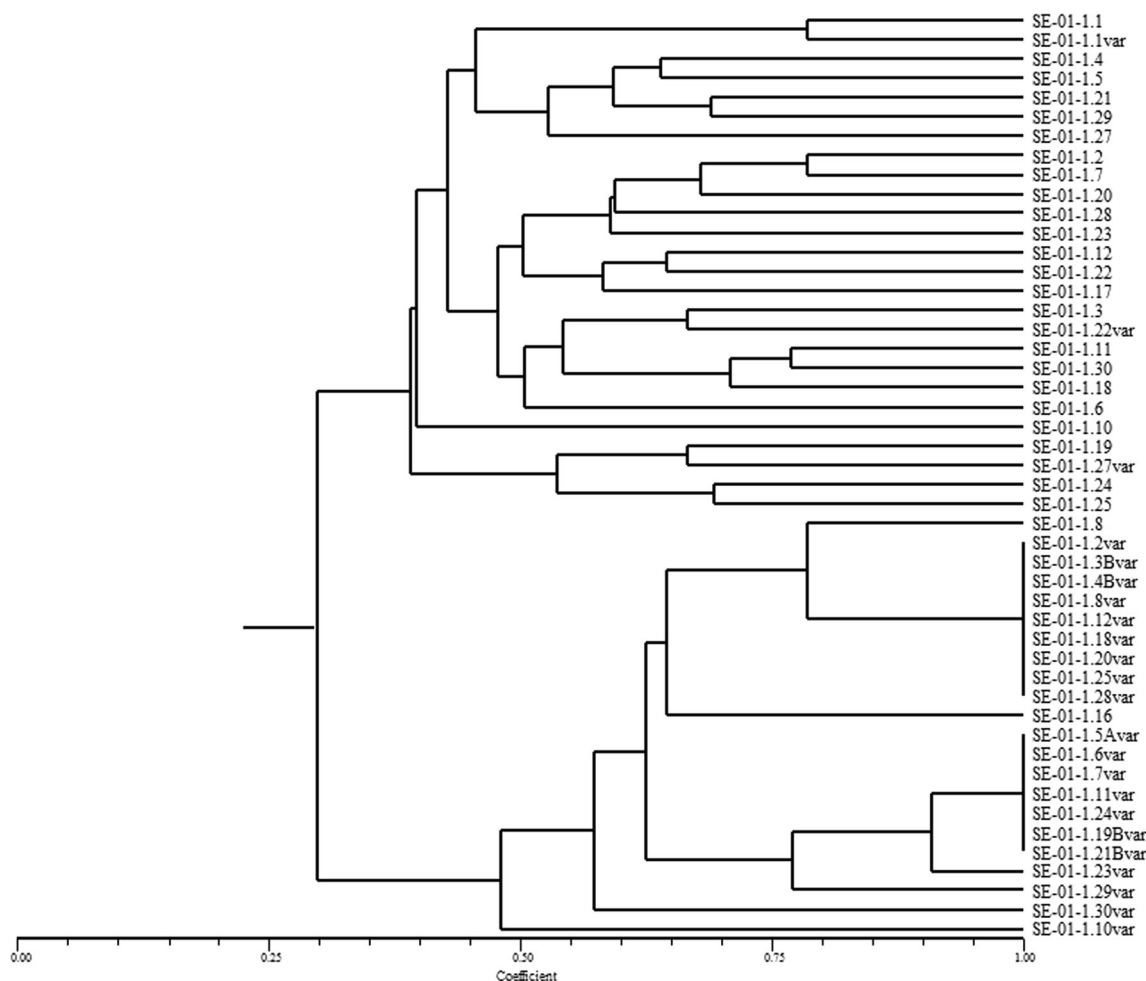
Fig. 3 Dendrograms showing the clustering patterns of chestnut traditional varieties vs. rootstocks

degree of genetic similarity to these dominant genotypes. This similarity may be the result of the germination of a nut from an original variety that, due to good fruit traits, was selected as a new variety. Such an observation was previously described in chestnut from southern Spain, a region that favours short juvenile period of the species and with high ability of farmers to practice grafting (Martín et al. 2009). The resemblance between these groups of varieties could indicate that they were brought from other areas, possibly as cutting material, and

Table 3   AMOVA analysis showing the proportion of genetic variation due to differences among rootstocks vs. varieties

Source of variation	df	Variance components	% Variation	P value
SSRs				
Among populations	1	0.70784Va	22.02	<0.001
Within populations	98	2.51401Vb	77.98	<0.001
EST-SSRs				
Among populations	1	0.52417Va	17.98	<0.001
Within populations	98	2.39143Vb	82.02	<0.001

sexual reproduction processes have led to the current situation. Results obtained with STRUCTURE could support this hypothesis, since varieties from Sevilla and Malaga were grouped together. Processes of this nature were also proposed for the evolution of wild olive (Muñoz et al. 2015). In addition, the fact that one of the rootstocks (SE-01-1.08) was included in the group of varieties suggests that this process could operate in both directions. Conversely, those varieties clearly different from the dominant groups could have emerged from rootstock population, and the best genotypes were used for grafting.

The EST-SSR loci results showed considerably lower polymorphism and diversity than that detected using genomic SSRs in chestnut, in agreement with reports for other plant species (Scott et al. 2000; Woodhead et al. 2005). Nevertheless, this low variation in EST-SSRs contrasts with their efficient discrimination capability for male catkin identification. Thus, the EST-SSR dendrogram separated the individuals according to the presence of astaminate, mesostaminate and longistaminate catkins. Such a result was unexpected because it was speculated that strong local
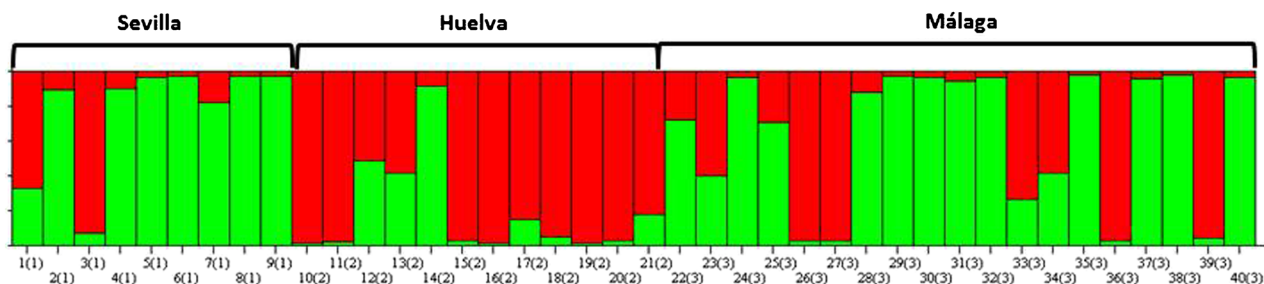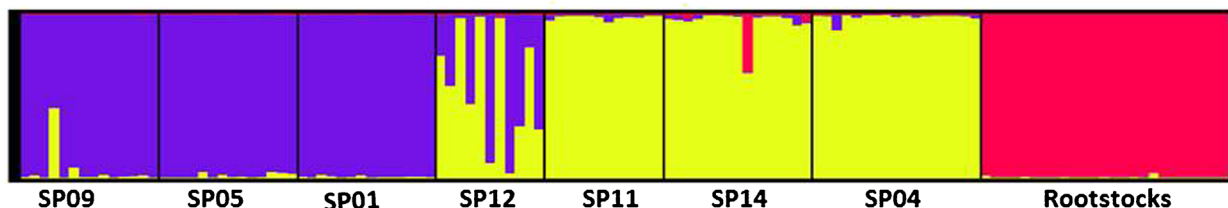
a *K*=2

b *K*=3

Fig. 4 Bayesian clustering of the chestnut accessions obtained with STRUCTURE software (Pritchard et al. 2000). **a** Genetic relationship among traditional varieties for *K* = 2. **b** Genetic relationship among rootstocks and chestnut populations for *K* = 3

selection due to human management would mask the possible signal of functional markers (Martín et al. 2016). Nevertheless, some of these markers demonstrated significant differences in the level of differentiation among chestnut populations from contrasting climatic environments in relation to bud burst, with northern populations flushing and forming winter buds later, and growing more than Mediterranean populations (Martín et al. 2010).

Our results highlighted that the chestnut production system is characterized by a complex structure and considerable genetic diversity, confirming results of previous studies on chestnut populations (Martín et al. 2012; Mattioni et al. 2013) and traditional varieties (Gobbin et al. 2007; Martín et al. 2009; Pereira-Lorenzo et al. 2010). Likewise, results of the present study showed that rootstocks harboured a high level of diversity, not previously described, and not contained in the genetic information from populations and varieties. In this respect, the most remarkable finding was the genetic integrity found between rootstocks and traditional varieties. The results indicate that rootstocks come from sexual reproduction and constitute a genetic population that harbour a high genetic diversity. Furthermore, only three varieties (SE-01-1.1var, SE-01-1.22var and SE-01-1.27var) could have emerged from this population. Considering the advanced age of the trees, it could be assumed that the rootstock population is native, or at least adapted to the area.

Furthermore, rootstocks and populations displayed different genetic composition. Thus, STRUCTURE analysis detected a clear structure among rootstocks and populations, showing absence of admixture between them (see Q admixture value in Online Resource 1). Likewise, different alleles were detected in these materials, including 23 private alleles in the rootstocks and 16 in the populations (data not shown). Similarly, a clear structure was also detected in the varietal germplasm, where Bayesian analysis evidenced that traditional chestnut varieties from Sevilla were clustered with those from Malaga, whereas varieties from Huelva had a definite identity. Nevertheless, despite being grouped with varieties from Malaga, seven different alleles not catalogued previously in southern material were found in varieties from Sevilla (Martín et al. 2009).

It is assumed that the wise use of genetic resources in trees maintained on farm is one of the real options available to support sustainable production systems (Graudal et al. 2014; Alfaro et al. 2014). Nevertheless, there is a substantial lack of field studies on the extent of conservation activities and on the reasons for maintaining landraces. It is widely accepted that without monitoring, it is not possible to verify effectiveness of such conservation (Schwatz et al. 2006). In this respect, this study provides valuable baseline data concerning the complex genetic relationships between wild and cultivated chestnuts that should contribute to understanding the human role in the management of the species, expand capacities to manage its genetic resources and open the possibility to generalize this approach to the rest of orchards in the Mediterranean basin. Furthermore, temporal changes in the reported results on the amount of diversity and its distribution in space could be assessed as relevant state indicator in these chestnut production systems. According to Aravanopoulos (2011), a frequency of one evaluation per decade should be adequate, given the current levels of anthropogenic exploitation and environmental changes. Nevertheless, taking into account that chestnut

orchards are highly human-managed, we consider that these measurements should be conducted in a shortened period of time. Furthermore, the advance age of the farmers that are the real maintainers of these traditional systems supports this fact (Martín et al. 2007).

On farm conservation of traditional chestnut varieties is dynamic and sustains the ongoing evolution of the crop, simulated by human management of varietal mixtures, graft exchange and mutations. Nevertheless, the implication of the public administration to encourage the producer associations and small farmers to maintain this system is necessary, complementing it with strategies of ex situ maintenance of this germplasm. At regional scale, the Natural Park of Sierra de Aracena y Picos de Aroche (Huelva province) is conducting an initiative to establish an *arboretum* with the most important genotypes from this region. The traditional varieties identified in this study will be included in it. Furthermore, these genotypes will be incorporated in a core collection at national level within the research project AGL2013-48017-C2-1-R from the Spanish Ministry of Economy and Competitiveness. Finally, our results demonstrate that rootstocks could constitute an unexploited reservoir of variation that would be valuable to counteract stress factors and future and unpredictable environmental changes in the species.

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