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Research Article

Genetic characterization of Greek chestnut (*Castanea sativa* Mill.) germplasm collections in Parnon mountain

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Abstract

The European chestnut (*Castanea sativa* Mill.) is a unique species located in Europe and the Mediterranean countries. In Greece, chestnuts are widely spread among 28 districts of the country due to their diverse usages by humans (wood production, chestnuts, mushrooms, honey), while they can be geographically placed, based on the nuts' morphological diversity in 6 main populations located in Kozani, Pilio, Karpenisi, Parnon, Lesvos and Crete. In this study, 56 trees from 3 different areas (Vamvakou, Varvitsa, Karyes) located in the west Parnon mountain region, were collected and subjected to genetic characterization with seven specific SSR molecular markers, in order to assess their genetic structure. The studied chestnuts exhibited a generally low level of genetic differentiation, with most of the genetic diversity residing within individual trees rather than being partitioned among different collection sites. Also, Karyes was not strongly genetically distinguished from Vamvakou and Varvitsa but the regions of Vamvakou and Varvitsa appeared to be genetically closely related compared to Karyes. The identified polymorphic microsatellite loci (especially CsCAT16 and CsCAT3) could be valuable tools for further, more detailed genetic studies of these chestnut populations.

Keywords: *Castanea sativa*; genetic diversity; local chestnut cultivars; molecular analysis; SSR markers

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Introduction

The genetic diversity found in traditional varieties and modern cultivars, crop wild relatives and other wild plant species that can be used for food and agriculture nowadays or in the future, is referred to as plant genetic resources for food and agriculture (PGRFA) (FAO, 1997). These resources serve as the starting point for the creation of new cultivars, which can be accomplished through traditional plant breeding or biotechnology. PGRFA are a source of genetic adaptation that serves as a safeguard against potentially detrimental environmental and economic change, whether they are utilized directly by farmers as a raw material or by plant breeders. Long-term global food security is seriously threatened by the depletion of these resources. Thus, although underestimated, there is essential necessity to preserve and use PGRFA as a buffer against an uncertain future (FAO, 1997; Hasan *et al.*, 2015; Salgotra *et al.*, 2023). In this aspect, local (indigenous) varieties or populations of all plant species constitute a treasure trove of plant genetic resources for local regions, but also for the countries. They constitute an evolving genetic material, the safeguarding of which is considered of paramount importance, both for the safeguarding of biodiversity and for the heritage and history of each place (Hasan *et al.*, 2015).

The European chestnut (*Castanea sativa* Mill.) is widely distributed throughout the Mediterranean region, from the Caucasus to Southern England (Botta *et al.*, 2001; Casasoli *et al.*, 2001). It is considered the only native European species of the Castanea genus and holds significant economic and ecological importance, especially in mountainous regions (Torello Marinoni *et al.*, 2013). The genetic diversity of the European chestnut has been assessed in various European natural (wild) populations, as well as in cultivated varieties (Fineschi *et al.*, 1994; Vilani *et al.*, 1994; Pereira-Lorenzo *et al.*, 1995; Vilani *et al.*, 1999). The general picture that has emerged is that the commercial cultivation of chestnuts has caused a steady decrease in genetic diversity. However, there are still areas that constitute an important source of genetic diversity, which can play an important role in the preservation of the biodiversity and adaptability of chestnut (Casasoli *et al.*, 2001).

Only the recent years, there has been a growing interest in the cultivation of chestnuts, both as an arboricultural and as a forestry species. This fact created the demand for selected chestnut varieties and therefore methods were developed for the characterization and identification of these varieties. The identification of chestnut varieties was traditionally based on morphological characteristics, the expression of which is, however, significantly influenced by developmental and environmental factors. For these reasons, the development of methods for genetic identification of chestnut populations became necessary (Botta *et al.*, 2001). In an effort to evaluate the genetic diversity and structure between and within European chestnut populations a few studies have been conducted in western, central and eastern areas of Europe, including Greece and Turkey. Fineschi *et al.* (2000) studied natural and naturalized chestnut popoulation from Turkey, Portugal, Spain, France and Italy. According to results the distribution of the cpDNA haplotypes revealed low geographical structure of the genetic diversity of chestnuts revealing the strong human impact on this species. Moreover, Mattioni *et al.* (2008) who assessed the genetic structure of sweet chestnut in five European countries (Italy, France, Spain, Greece, and UK) found the existence of five distinct gene pools across the study area, three in Greece, one on the northwestern coast of the Iberian Peninsula and a large gene pool covering the rest of the Mediterranean basin, that rules out the migration of the chestnut from Turkey and Greece to Italy. The homogeneous gene pool observed in Italy and Spain could have been originated from common refugia along with human-mediated colonization. Moreover, in a latter study (Mattioni *et al.*, 2013) that examined the genetic relationships among the populations from Turkey and those from Greece and western Europe, it was found that the population structure showed a genetic divergence between the eastern (Greek and Turkish) and western (Italian and Spanish) populations and that two gene pools and a zone of gene introgression exist in Turkey. Fernández-Cruz and Fernández-López (2016) found a Northern Iberian Peninsula cluster that included the northern and northwestern Spanish populations and a Mediterranean cluster that contained central and southern Spanish, Italian and Greek populations. According to authors, the results of the study suggest that the current genetic structure of sweet chestnut is explained by the existence of several refugia during

Pleistocene and by human activity that changes the structure of the populations. In an extensive study regarding wild populations and natural or naturalized chestnuts sampled in various countries (Spain, Italy, Portugal, France, England, Greece, Slovakia, Hungary, Romania, Bulgaria, Georgia, Russia, Turkey and Azerbaijan), using six polymorphic microsatellite markers, three main gene pools and a significant genetic barrier separating the eastern from the central and western populations were identified. Areas with high priority for genetic conservation were indicated in Georgia, eastern Turkey, and Italy (Mattioni *et al.*, 2017). Sweet chestnut populations from Central Europe and the western part of the Balkan Peninsula, utilizing ten polymorphic nuclear microsatellite markers were assessed by Poljak *et al.* (2017). Authors found the existence of three genetically and geographically distinct and well-defined groups of sweet chestnut populations, two in the northern part of the studied area and one in the southern. According to authors the results indicated that the genetic structure of sweet chestnut populations in Central Europe and the western part of the Balkan Peninsula is the result of both natural colonization events and significant and lengthy human impact.

In Greece, chestnuts are widely spread among 28 districts of the country due to their diverse usages by humans (wood production, chestnuts, mushrooms, honey), while 6 main populations have been recognized (Kozani, Pilio, Karpenisi, Parnon, Lesvos and Crete), based on their geographical distribution rather than genetic differences (Diamandis, 2010). To date, a comprehensive genetic identification of these six recognized indigenous chestnut populations has not been fully implemented in Greece, in order to determine the genetic variability, both between populations and within populations, in their places of origin. According to Aravanopoulos *et al.* (2001), before implementing strategies and practices for gene conservation, both at national and European level, the size and structure of the genetic diversity of natural (autochthonous) chestnut populations must be established. In Crete, the four most well-known chestnut cultivars (Strovliani, Rogdiani, Koutsakera, and Katharokastania) were evaluated for their genetic diversity. The analysis revealed twenty-two distinct genotypes, indicating that each cultivar is actually a multiclonal variety. The substantial genetic variation observed suggests that the Cretan chestnut germplasm represents a valuable gene pool deserving further study. Such investigations will enhance the understanding of its true genetic value and support efforts aimed at its conservation and sustainable use in pre-breeding and breeding programs (El Chami *et al.*, 2021). Kampourolias *et al.* (2021) studied the genetic diversity and fruit morphological characteristics of chestnut trees grown in five neighboring Arcadian villages Kastri, Platanos, Agios Petros, Kastanitsa and Doliana. Their findings revealed low genetic variability among the trees and consistent fruit characteristics, indicating that these chestnut populations may belong to a distinct local genotype warranting further research.

Greece held the seventh position worldwide in chestnut in shell production in the decade 2013-2023 (FAOSTAT, 2025). The Peloponnese region is recognized as one of the most suitable areas for chestnut cultivation and holds the third-highest production level within the country (ELSTAT, 2025). It is renowned for its rugged mountain terrain, particularly the Parnon mountain range, where traditional orchards continue to support chestnut cultivation and where the Parnon chestnut population is located. The Parnon mountain region, also known as Parnonas or Malevos, is the most extensive mountain complex in Peloponnese, in Greece. With a range of 90-100 kilometers, Parnon is renowned for its diverse landscapes, including deep gorges, valleys, mountain peaks, caves, waterfalls, and springs. It is characterized as a rich habitat of high ecological value due to its diverse flora, including coniferous forests with Kefalonian fir, black pine, rare cedar and deciduous trees like chestnut, oak, and plane. This mountain provides a high degree of genetic isolation and climatic conditions favorable for chestnut growth.

Today, the Parnon region still harbors a rich gene pool of local chestnut landraces, highlighting the need for renewed efforts to identify, characterize, and utilize the local Parnon sweet chestnut population. Our hypothesis is that a detectable level of genetic diversity among chestnut trees in the Parnon mountain region exists, which can be characterized using SSR markers, and that this diversity is structured geographically In this effort, 56 individual trees from 3 different areas, (Vamvakou, Varvitsa, Karyes) of the prefecture of Laconia located in the west Parnon mountain region, were collected in order to assess the extent of genetic diversity using SSR markers, to analyze the genetic relationships among individual trees and identify any potential population structure and finally, to investigate the correlation between genetic diversity and geographic location of the sampled trees.

Materials and Methods

Description of the study site and sampling design

Plant material from a total of 56 individual trees were collected during the year 2023 and their GPS coordinates are shown in Table S1. The trees were spread over three areas of Laconia prefecture (Vamvakou, Varvitsa, Karyes) located in the west Parnon mountain region (Figure 1). During field work, 10 to 15 leaves were collected and preserved accordingly, transferred to the laboratory and stored at -20°C. Due to the lack of prior knowledge about the genetic relationships among the sampled trees and the sampling strategy employed, we decided to treat each tree as an individual unit. This approach allowed us to capture the maximum possible genetic variation within the sampled area.

The sampled trees were located in traditional orchards that have been cultivated and maintained for several generations. While specific planting dates and parentage records are lacking, local knowledge suggests that some orchards may contain a mix of trees propagated from seed and grafted varieties. There is a tradition of exchanging plant material among orchard owners in the region, which could have influenced the genetic makeup of the chestnut population. Efforts were made to sample trees that appeared to be distinct individuals based on their spatial separation within the orchards, but we cannot definitively rule out the possibility of some relatedness among them. However, we recognize the potential limitations and implications for the interpretation of our results.

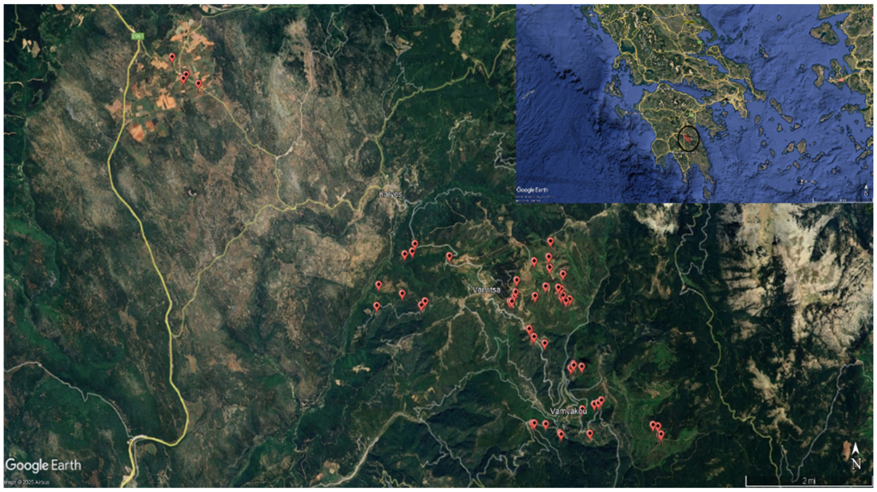

Figure 1. Location of the sampling sites for the 56 collected chestnut trees among the three areas of Vamvakou, Varvitsa and Karyes. Geographical position of the investigated area in Greece

DNA extraction and quantification

Samples were grinded into fine powder, with the addition of liquid nitrogen, using porcelain mortar and pestle. Total genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) with minor modifications. DNA quality and quantity were determined by 1% agarose gel electrophoresis and by Quawell UV-Vis Spectrophotometer (Q3000, Quawell Ltd).

PCR amplification and capillary electrophoresis

The seven chosen SSR markers (Table 1), were specifically selected from a broader set based on their consistently high levels of polymorphism and informativeness as demonstrated in prior studies of *Castanea* *sativa* (Buck *et al.*, 2003; Marinoni *et al.*, 2003; Bini *et al.*, 2023), were employed. These markers exhibit a high number of alleles per locus and high heterozygosity values, ensuring that even with a limited number, they capture a significant portion of genetic variation. This strategic selection prioritizes quality over quantity, aiming for markers that are highly discriminative. Also, a smaller set of highly informative markers can often provide sufficient resolution for addressing the specific objectives of this study, focusing on differentiating between closely related populations or identifying distinct genetic clusters within a relatively small geographic area. Forward primers were labeled with a fluorescence tag (5-FAM, HEX, TAMRA and ROX) (Eurofins Genomics, Ebersberg, Germany). PCRs were performed on a final volume of 25 μL containing 20 ng of genomic DNA, 1 U Kapa Taq DNA polymerase, 1x of the supplied PCR buffer with $MgCl_2$ (1.5 mM), 0.8 μM of each primer and 0.4 mM of dNTPs. PCR reactions were performed using a SureCycler 8800 thermocycler (Agilent Technologies, California, USA) under the cycling profile: first step at 94 °C for 4 min, followed by 35 cycles segmented in 40 s at 94 °C, 40 s at varied temperature based on primers (Table 1) and 40 s at 72 °C, and a final extension at 72 °C for 5 min. 4 μL of each amplified PCR products were checked in 3% agarose gels in order to validate the presence of the expected amplified band, before their separation on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Inc. (ABI), Carlsbad, CA, USA), using a performance-optimized polymer (POP7), together with the GeneScanTM-600 LIZ Size Standard (ABI) as internal size standard. Microsatellite analysis was performed with Geneious Prime 2019.2 software (Biomatters, Auckland, New Zealand).

Table 1. List of primer sequences for microsatellite loci, with corresponding annealing temperature and length range of the amplicons

Primer name	Primer sequence (5' → 3')	Repeat Motif	Annealing temperature (°C)	Product size	Reference
CsCAT1	F:GAGAATGCCCACTTTTGCA R: GCTCCCTTATGGTCTCG	$(TG)_5TA(TG)_{24}$	50	190–224	(Marinoni *et al.*, 2003)
CsCAT16	F: CTCCTTGACTTTGAAGTTGC R: CTGATCGAGAGTAATAAAG	$(TC)_{20}$	50	130–147	(Marinoni *et al.*, 2003)
CsCAT17	F:TTGGCTATACTTGTTCTGCAAG R: GCCCCATGTTTTCTTCCATGG	$(CA)_{19}A(CA)_2$ $AA(CA)_3$	58	138–160	(Marinoni *et al.*, 2003)
CsCAT3	F:CACTATTTTATCATGGACGG R:CGAATTGAGAGTTCATACTC	$(AG)_{20}$	50	208–258	(Marinoni *et al.*, 2003)
CsCAT6	F: AGTGCTCGTGGTCAGTGAG R: CAACTCTGCATGATAAC	$(AC)_{24}AT(AC)_4$	50	158–194	(Marinoni *et al.*, 2003)
QpZAG110	F: GGAGGCTTCCTTCAACCTACT R: GATCTCTTGTGTGCTGTATTT	$(AG)_{15}$	53	210–230	(Steinkellner *et al.*, 1997)
QpZAG119	F:GATCAACAAGCCCAAGGCAC R:GGCATGTGTATTGAAAGCTGTA	$(GA)_{24}$	55	210–223	(Steinkellner *et al.*, 1997)

Data analysis

Null alleles frequencies were calculated from loci information through ML-Null Freq software using the Hardy-Weinberg test for heterozygote deficiency (Kalinowski and Taper, 2006) and FreeNA computer program that estimates null allele frequencies for each locus and population analysed following the Expectation Maximization (EM) algorithm (Chapuis and Estoup, 2007). The number of alleles (Na), the number of effective alleles (Ne), the Total Expected Heterozygosity (Ht), the observed (Ho) and expected (He) heterozygosity, the number of migrants (Nm), the fixation index (Fst) and the Shannon index (I) were computed using GeneAlEx 6.5 (Steinkellner *et al.*, 1997; Kalinowski *et al.*, 2006). A Mantel test, through GeneAlEx 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012), was performed to assess the correlation between genetic profiles based on pairwise genetic distances between our samples and geographic distances between sampling locations calculated from latitude and longitude coordinates, with a value of 999 permutations (Mantel, 1967). Genetic distance matrices were calculated and implemented for further genetic analyses including Analysis of Molecular Variance (AMOVA) and Principal Coordinates Analysis (PCoA) using GeneAlEx 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). The genetic distance matrix was also used for the construction of the UPGMA tree with MEGA X (Kumar *et al.*, 2018).

Results

In total, 50 alleles were detected in the 7 microsatellite loci. The number of alleles per locus varied between three and twelve (average of 7.1 alleles per locus). Results from Hardy-Weinberg test revealed high p values ranging from 0.9950 (CsCAT17) to 1.0000 (CsCAT3, CsCAT1, QpZAG110 and CsCAT6) for all primers used in this study, indicating the absence of null alleles for all loci and the efficiency of the chosen primers. These results were also validated with FreeNa program where the null allele frequencies using the EM algorithm were estimated as 0.00000, using 10.000 bootstrap replications. The most polymorphic locus was CsCAT16 that presented an observed heterozygosity (Ho) of 0.934 followed by CsCAT3 and QpZAG119, which presented values of 0.924 and 0.845 (Table 2). Also, CsCAT16 and CsCAT3 had the highest values of effective number of alleles (Ne) of 5.128 and 4.098, respectively.

Table 2. Descriptive genetic parameters for each SSR locus in the 56 chestnut trees. The number of alleles (Na), the effective number of alleles (Ne), the total expected heterozygosity (Ht), the expected heterozygosity (He), the observed heterozygosity (Ho), the fixation index (Fst), gene flow (Nm), and the Shannon index (I) are detailed

Locus	Na	Ne	Ht	He	Ho	Fst	Nm	I
CsCAT16	6.000	5.128	0.866	0.801	0.934	0.075	3.070	1.675
QpZAG110	4.333	3.630	0.741	0.675	0.838	0.089	2.547	1.275
CsCAT6	4.000	2.144	0.564	0.533	0.315	0.054	4.376	0.928
CsCAT3	6.000	4.098	0.783	0.747	0.924	0.045	5.257	1.521
CsCAT17	4.667	3.124	0.738	0.622	0.754	0.157	1.345	1.210
QpZAG119	2.667	2.066	0.518	0.515	0.845	0.005	52.653	0.763
CsCAT1	4.333	3.466	0.740	0.681	0.786	0.080	2.879	1.267
Mean	4.571	3.380		0.653	0.771	0.072	10.304	
SE	0.356	0.293		0.030	0.053	0.018	7.074	

The fixation index (*Fst* = 0.018) shows a low level of differentiation between the collected trees. The gene flow (*Nm*) ranged between 1.345 for CsCAT17 and 52.653 for QpZAG119 (Table 2). According to AMOVA, 92% of the total diversity resides within individuals and 8% among individuals.

The UPGMA dendrogram (Figure 2) distributed the collected chestnut individuals across five main clusters and some individuals were not clustered (Kar48, Vam7, Vam8, Varv35 and Varv31). In general, cluster I contains chestnut individuals mainly from Vamvakou and Varvitsa regions, suggesting that the genetic divergence within this group is likely small, meaning they are more genetically uniform. Cluster II contains a mix of chestnut individuals from Karyes and Varvitsa which share a strong genetic similarity. Cluster III contains only individuals from Karyes region and cluster V contains only individuals from Vamvakou region, suggesting that they might represent genetically coherent populations with moderate genetic variation. Finally, cluster IV contains a mix of chestnut individuals from two regions that form two subclusters, one with individuals from Vamvakou and one from Varvitsa, indicating that they may belong to the same genetic lineage with minor variations. The Mantel test did not reveal any correlation between genetic and geographic distances, as shown in Figure S1 (Supplementary).

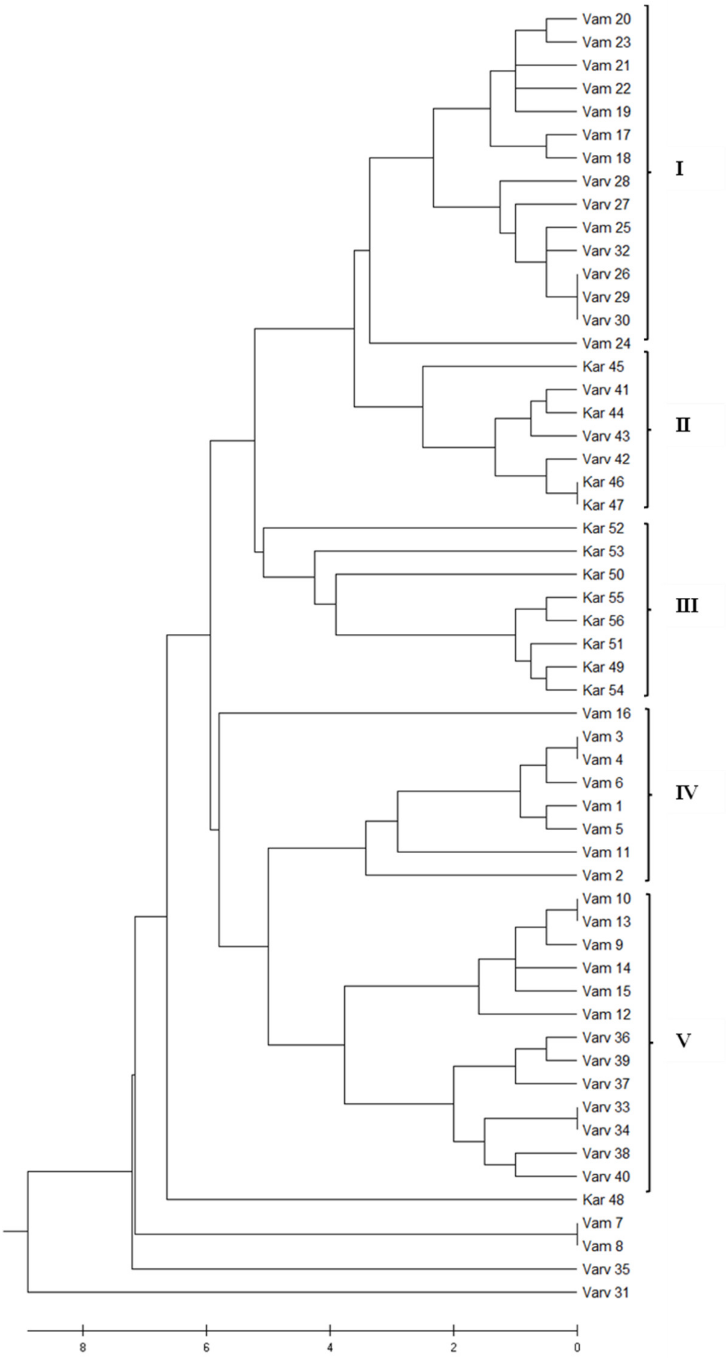

Figure 2. UPGMA dendrogram of the studied 56 chestnut trees based on 7 SSR markers. Five main genetic clusters were formed: Clusters I and IV showed a mix of individuals, suggesting genetic mixing or shared ancestry between the Vamvakou and Varvitsa regions (Cluster I) and Vamvakou and Varvitsa again (Cluster IV, with two subclusters). In contrast, Clusters II, III, and V revealed more geographically specific groupings: Cluster II combined individuals from Karyes and Varvitsa, while Cluster III was exclusively Karyes and Cluster V was solely Vamvakou, indicating potentially more coherent and distinct genetic populations in these areas. A few individuals (Kar48, Vam7, Vam8, Varv35, Varv31) did not clearly fit into any cluster

A PCoA was carried out where the first two components accounted for 47.67% of the total variance. As seen in Figure 3, several small groups were formed that agree with the clustering of the groups from the UPGMA dendrogram. Most of the groups contained mixed chestnut individuals, except for the one group that contained only individuals from Varvitsa and one that contained only individuals from Vamvakou and together, these two groups formed cluster I of the dendrogram.

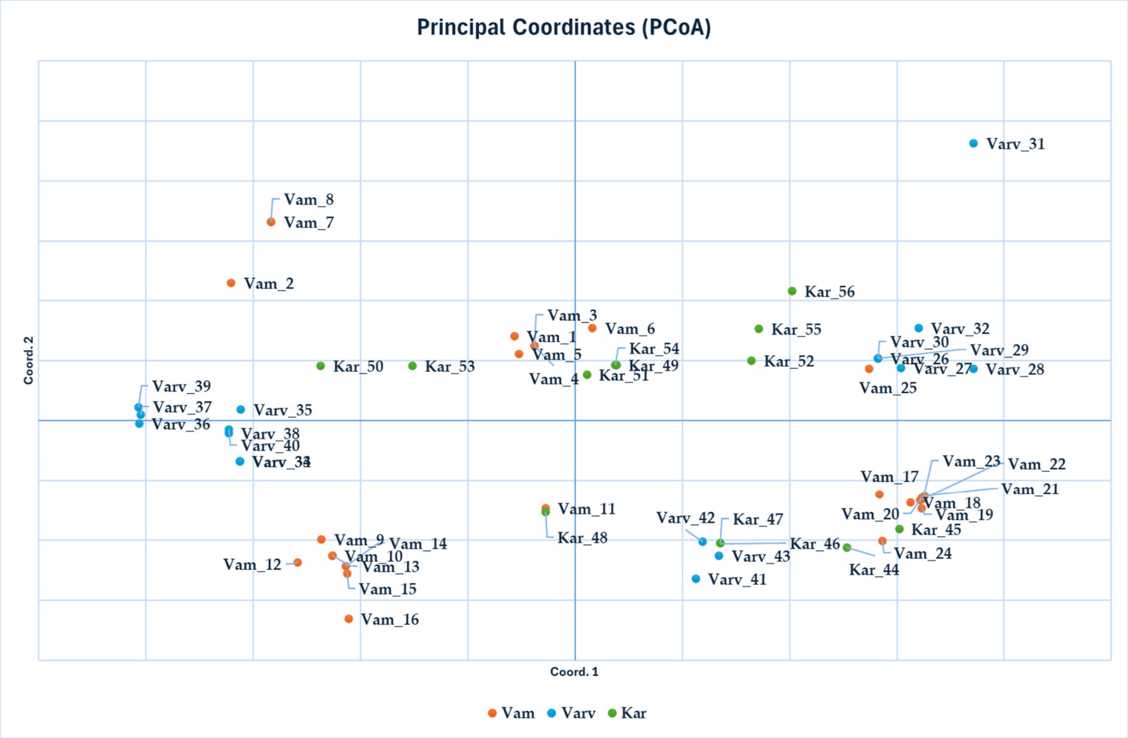

Figure 3. Principal Coordinates Analysis for SSR markers of the 56 collected samples showed the same formation of clusters as in the UPGMA dendrogram

Diversity statistics with respect to the sampling regions are displayed in Table 3. Regarding the pairwise matrices of Nei's unbiased genetic distances and Nei's unbiased genetic identity, the maximum distance was detected between Vamvakou and Karyes (0.344). Conversely, the lowest distance was observed between Vamvakou and Varvitsa (0.182). The greatest genetic identity was found among the Vamvakou and Varvitsa chestnuts (0.833), with the lowest one between Vamvakou and Karyes (0.709). Regarding the highest observed heterozygosity value occurred in Vamvakou (*Ho*=0.850) and the lowest in Karyes (*Ho*=0.670). The values of expected heterozygosity are similar for all three regions, along with the number of effective alleles.

Table 3. Genetic diversity statistics for the three regions sampled

Pop	N	Na	Ne	I	Ho	He	F
Vamvakou	25	5.000	3.534	1.286	0.850	0.667	-0.301
Varvitsa	18	4.143	3.399	1.247	0.792	0.680	-0.163
Karyes	13	4.571	3.205	1.169	0.670	0.614	-0.110

N: number of samples; Na: number of alleles; Ne: number of effective alleles; I: Shannon Index; Ho: observed heterozygosity; He: expected heterozygosity; F: inbreeding coefficient

AMOVA partitioned the genetic diversity as 16% between regions and 84% within regions (Table 4). Moreover, a high overall *Nm* value (2.804) was noticed, with *Fst* showing a considerably low value of 0.082 (*p*-value < 0.001). The pairwise *Fst* revealed the lowest value between Vamvakou and Varvitsa (0.038), whereas the highest *F* index was noticed between Vamvakou and Karyes trees (0.074).

Table 4. Analysis of Molecular Variance (AMOVA) for the three regions sampled

Source	df	SS	MS	Est. Var.	%	PhiPT	*p*-Value
Among Pops	2	41.812	20.906	0.900	16%	0.161	0.001
Within Pops	53	248.991	4.698	4.698	84%		

df: degrees of freedom; SS: sum of squares; MS: mean squares; Est. Var.: Estimated Variance

Discussion

Chestnut cultivation has been and still is an important feature of the Parnon mountain region, contributing to its natural beauty, ecological diversity, and local culture and economy. So far, there is not a systemic genetic diversity study of the local Parnon chestnut trees. The study and protection of chestnut trees is crucial for preserving unique genetic variations that offer special characteristics that allow them to adapt to different environments and to create high quality end products (Torello Marinoni *et al.*, 2013). The present work offers a first genetic assessment of the chestnut trees located in three specific regions in Parnon by implementing seven SSR primers.

Although the individuals were collected in a restricted geographic area, a high number of 50 alleles and regarding the genetic diversity that was found within loci, the seven microsatellite loci exhibited a moderate level of allelic diversity, with an average of 7.1 alleles per locus (ranging from 3 to 12). This suggests that a reasonable level of genetic variation has been assessed. These findings agree with the results from studies with greater number of studied trees and SSR molecular markers (Torello Marinoni *et al.*, 2013; Poljak *et al.*, 2017), indicating that the SSR markers selected for this study were very efficient in analyzing the presence of the genetic diversity, along with the high *p*-values from the Hardy-Weinberg test and the FreeNa analysis (null allele frequencies estimated at 0.00000) that strongly indicated that null alleles are not a significant concern for these loci. This validated the reliability of the chosen primers for the genetic analysis of the samples. Also, CsCAT16 and CsCAT3 standed out as the most polymorphic loci, displaying the highest observed heterozygosity (Ho) and effective number of alleles (Ne). These loci can be characterized as highly informative for future genetic differentiation studies on chestnut.

Regarding the genetic differentiation and gene flow among all collected trees, based on the low fixation index (Fst = 0.018), it was indicated that a minimal level of genetic differentiation exists among all the collected chestnut trees. This finding suggests the presence of a high degree of genetic similarity across the entire sampling site. Also, the relatively high overall gene flow (Nm), ranging from 1.345 to 52.653 across loci, further supported the idea of limited genetic divergence among the sampled trees that could be attributed to substantial historical or ongoing gene exchange. AMOVA has shown high within-individual variation (92%) that indicates that most of the genetic differences observed in our dataset occurred between the two copies of the genome within a single individual, leading to the fact that individuals are highly likely to be heterozygous (carrying two different versions or alleles). Also, AMOVA revealed low differentiation within sampling locations, suggesting that individuals within the same sampling location are genetically quite similar to each other and there isn't much structured genetic variation distinguishing between them. Furthermore, AMOVA revealed a relatively low percentage among-individual differentiation (8%), indicating that there is only a minor degree of genetic difference when you compare one individual to another. While there are some genetic distinctions between individuals, they are considerably smaller than the differences found within each individual. But although the small percentage (8%) still some level of genetic differentiation among individuals can be found. This fact can be attributed to: a) Limited gene flow: Some restriction in the movement of genes between individuals (though not enough to create strong population structure), b) Recent divergence: The groups might have diverged relatively recently, and significant genetic differences haven't yet accumulated, c) Specific mating systems: Certain mating patterns could lead to slightly more related individuals within groups, and finally, d) Selection pressures: Weak or localized selection pressures could be contributing to some differentiation. The AMOVA findings strongly suggest a lack of strong population structure or differentiation, as most of the variation is within individuals sharing a common genetic origin and there is not much genetic "partitioning" among different groups. This observation is consistent with prior studies that have consistently shown that most genetic variation is found within individuals and sharing a common genetic origin (Lang and Huang, 1999; Quintana *et al.*, 2015). Research on central European and Balkan chestnuts further supports these findings, highlighting that the majority of genetic diversity resides within populations (Lang and Huang, 1999; Quintana *et al.*, 2015).

The chestnut trees collected from the three regions of Vamvakou, Varvitsa and Karyes demonstrated high values of observed heterozygosity (mean Ho=0.77) and are in accordance with findings from similar studies regarding Spanish (Quintana *et al.*, 2015), Italian (Martín *et al.*, 2017) and Cretan (El Chami *et al.*, 2021) cultivars (Ho = 0.5, Ho = 0.6 and Ho = 0.7, respectively).

According to the cluster analysis, five main groups were formed that mostly contained mixtures of the collected chestnut individuals, except for cluster III that contained only trees from Karyes. The formation of clusters III (Karyes) and V (Vamvakou) suggested these regions might represent more genetically coherent populations with moderate internal variation. Low genetic distances and high genetic identity among individual trees from Vamvakou and Varvitsa, that formed subclusters within cluster I and cluster V, can be justified by the fact that the cultivated trees are located in traditionally managed orchards and without prior knowledge of pedigree information, propagation methods including both seed propagation and grafting, cannot be excluded. This likely contributed to the genetic diversity observed. The maximum genetic distance was observed between Vamvakou and Karyes, suggesting a higher level of genetic divergence between these two regions compared to the Vamvakou-Varvitsa pair. Vamvakou exhibited the highest observed heterozygosity, while Karyes showed the lowest, suggesting differences in the levels of genetic diversity within these regions. Expected heterozygosity and the number of effective alleles were more similar across all the regions.

The Mantel test did not reveal any correlation between genetic and geographic distances, similar to Cretan and Spanish cultivars (El Chami *et al.*, 2021; Quintana *et al.*, 2015). These findings can be attributed to the origin of the collected trees from planted orchards with short geographical distances. The AMOVA results, showing that 84% of genetic variation is within regions, strongly indicate a statistically significant but still relatively low level of genetic differentiation among the three regions, due to the use of consistent cultivation and domestication methods throughout the area investigated over long periods. The overall Nm value of 2.804 among regions suggests a moderate level of gene flow, which is consistent with the relatively low Fst value (0.082), while the pairwise Fst values further highlight the close relationship between Vamvakou and Varvitsa (lowest Fst) and the greater differentiation of Karyes from Vamvakou (highest Fst).

As indicated by Casasoli *et al.* (2001), the European chestnut's evolutionary trajectory is a product of both natural environmental changes and human interventions. This means that its genetic diversity is shaped by geographical distribution, historical events, environmental shifts, and long-term cultivation. As expected, this research revealed moderate levels of genetic diversity among the collected chestnut trees within the Parnon region compared to other studies of *Castanea sativa*. The same results were obtained from 54 chestnut plants sampled from 5 villages from the prefecture of Arcadia (Kampourolias *et al.*, 2021). This lack of diversity can be attributed to the sharing of material among local cultivators and the selection of cultivars based on desired traits, such as the production of large fruits, based on interviews with local orchard owners from which the samples were taken.

Despite the limitations that arise from the restricted number of markers used in this study (overestimation of gene flow or underestimation of isolation, difficulty in resolving hybridization or introgression, sampling variance), the careful selection of highly polymorphic and informative markers, combined with robust analytical methods (e.g., AMOVA, PCoA, UPGMA), allows for meaningful insights into the genetic diversity and broad population structure of chestnut within the scope of this study. Future studies could benefit from incorporating a larger number of markers, such as those derived from next-generation sequencing (e.g., SNPs), to gain even finer-scale resolution and a more comprehensive understanding of population dynamics. Although the number of SSR molecular markers employed was restricted, the resulting polymorphic bands provided an indication of the genetic diversity present in the sweet chestnut germplasm of the Parnon region. The observed lack of greater genetic diversity raises the possibility of a distinct local genetic profile in this area of Parnon. These plants could be considered as local sources of genetic variation that require preservation and protection since they are valuable genetic resources and heritage. In order to guide a conservation strategy and ensure sustainable use of natural resources, it may be considered essential to assess the factors contributing to the observed patterns of genetic variation and differentiation through further research (Lang and Huang, 1999). These findings provide a good baseline understanding of the genetic diversity and relationships among the sampled chestnut trees and regions, but further research with more markers or/and a different type of markers (e.g. SNPs) is required for in depth genetic analysis.

Conclusions

This study focused on the genetic characterization of sampled chestnut trees from orchards located in the west Parnon mountain region in Greece, which have local importance, implementing SSR markers. The Parnon mountain is a critical source of genetic diversity for chestnut trees, a resource essential for their survival. These trees are precious genetic resources for a future breeding plan and conservation strategy. In summary, our study found that individual chestnut trees are genetically diverse internally and there is limited genetic differentiation among individuals from the three sampling locations. While there is some evidence for genetic structure, particularly distinguishing Karyes from Vamvakou and Varvitsa, the overall differentiation is not strong. The results suggest that there is likely weak or no strong population structure among the sampling locations. Our findings provide a snapshot of the genetic diversity among the sampled individual trees in the Parnon mountain region acknowledging the limitations of our study design and the lack of historical data regarding the orchards, and that further research with a more comprehensive sampling strategy across large geographic distances would be needed to fully characterize the genetic structure of the chestnut populations in this area. Furthermore, to protect this diversity, we must implement on-site conservation efforts. This includes sustainable forest management, protected areas, and promoting natural regeneration, enhanced by the application of reliable molecular markers. Moreover, further investigation is required through comparative analysis of the local germplasm with samples from other Greek regions and international cultivars.

Authors' Contributions

Conceptualization: IM; Data curation: IM and PM and LK and AX; Formal analysis: PM and LK and AX; Funding acquisition: IM; Investigation: IM and TS; Methodology: IM and PM and LK and AX; Project administration: IM; Resources: IM and LK; Software: LK; Supervision: IM; Validation: IM and LK and TS and PM; Visualization: IM and LK; Writing—original draft: IM and TS and LK; Writing—review and editing: IM and LK.

All authors read and approved of the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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