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Genetic Characterisation of Chestnut Cultivars in Crete

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Abstract: (1) Background and objectives: Cretan chestnut belongs to sweet chestnut (*Castanea sativa* Mill.) and has been historically associated with the lifestyle of rural communities with great economic importance. However, chestnut genetic resources in Crete have rarely been studied and assessed, while chestnuts are threatened by several anthropogenic factors. This study assessed the genetic variability of the Cretan sweet chestnut using 59 trees corresponding to the four best-known chestnut cultivars (Strovliani, Rogdiani, Koutsakera and Katharokastania). (2) Materials and Methods: The trees were evaluated using seven simple sequence repeat markers (SSRs): three nSSRs and four EST-SSRs. (3) Results: Genomic SSR results revealed notable genetic diversity in terms of expected heterozygosity, level of polymorphism and effective number of alleles. Moreover, in the four chestnut cultivars, twenty-two unique genotypes were identified, deeming each cultivar to be in fact a multiclonal variety. Genetic differentiation among cultivars was relatively low, though highly significant. Four different groups of synonymies were found: two homonymy groups in Katharokastania and Strovliani, six in Rogdiani and eight in Koutsakera. The cluster analysis and PCoA results reveal two main clusters, one corresponding to the Rogdiani cultivar and the other to Katharokastania, while the other two could not be assigned to a particular group. (4) Conclusions: The null hypothesis of single-clone genotype-to-cultivar correspondence was tested and could not be accepted.

Keywords: *Castanea sativa*; nSSR; EST-SSR; genetic diversity; synonyms; homonyms

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1. Introduction

Originating in the Caucasus and Asia Minor, where it was first domesticated and spread throughout southern Europe, sweet (or European) chestnut, *Castanea sativa* Mill., is the only native species of the *Castanea* genus in Europe [1]. During the Middle Ages, sweet chestnut was considered the "mountain cereal", and today it occupies three climatic sub-regions, growing from sea level up to 1800 m over a wide range of climatic conditions [2,3].

Historically, chestnut has been used as an important ingredient in many nutrient products due to its richness in various nutriments [4]. Moreover, chestnut trees have been distributed widely throughout mainly southern Europe, forming high-density forests in France and Italy, indicating the importance of this tree economically and environmentally [5]. Like many European forest species, chestnut has notable potential in reducing pollution and climate change effects [6]. The potential multi-use value of chestnut in the past is still valid today and new uses have proven to be economically important.

From a sustainable arboricultural point of view, it is crucial to combine increased productivity and competitiveness with the maintenance of biodiversity. Landraces and local varieties are saved simply because they fill ecological, cultural and local socioeconomic positions not occupied by modern varieties [2]. Thus, it is very important to assess the genetic diversity of traditional local varieties. However, assessing their diversity is very difficult, due to the absence of standard references, confusion of "variety" names (homonymy and synonymy) and the existence of multiclonal varieties as a result of the richness of chestnut genetic heritage [7,8].

Traditional chestnut varieties are characterised according to the geographical origin, morphology, ripening period and type of use [8]. In Crete, four traditional varietal names have been reported (Katharokastania, Koutsakera, Rogdiani and Strovliani); nevertheless, their classification is unclear due to the absence of pertinent studies.

This study reports the use of nSSR and EST-SSR markers for the identification of chestnut cultivars present in Crete. The null hypothesis of single-clone genotype-to-cultivar correspondence was tested. The main objective was to characterise the Cretan chestnut varieties genetically and to detect possible homonymies and synonymies.

2. Materials and Methods

2.1. Plant Material

Plant material of the four best-known and most widely spread Cretan chestnut varieties, Strovliani (S), Rogdiani (R), Koutsakera (K) and Katharokastania (Ka), was collected from the Chania region, Crete, during 2017. A total of 59 trees, each assumed to be one accession, were sampled and their GPS coordinates were registered (Table A1). The trees are located in chestnut orchards spread over seven main geographical areas: Elos (E), Milones (M), Floria (F), Palea Roumata (PR), Prases (P), Selli (S) and Sempronas (Sm) (Figure 1a). During field work, 15 to 20 leaves were collected and preserved in hermetically sealed plastic bags which were stored in an ice box and then transferred to a −80 °C fridge in the laboratory. Samples were grinded by liquid nitrogen using porcelain mortar and pestle prior to DNA extraction. Total genomic DNA was isolated according to the NucleoSpin® Plant II kit MACHEREY-NAGEL protocol. DNA quality and concentration were determined using a Thermo Scientific™ spectrophotometer Nanodrop™ 2000/2000 c.

2.2. Polymerase Chain Reaction (PCR) Amplification and Electrophoresis

Seven genomic labelled microsatellites (three SSRs and four EST-SSRs; Table 1), designed and used in previous *Castanea sativa* studies [9–13], were employed. The PCR was performed on a final volume of 23 µL containing 50 ng DNA, 0.5 U Taq DNA polymerase (Thermo Scientific™), the supplied buffer reaction with $MgCl_2$ (15 mM), 5 µM of each primer and 10 µM of dNTPs. A Bio-Rad DNA ENGINE DYAN (Bio-Rad) was used, programmed to follow: (1) a denaturation step (at 94 °C for 2 min, followed by 94 °C for 45 s); (2) annealing (30 s at the optimal temperature tested for *Castanea sativa* for each primer pair and ranging from 50 °C to 56 °C); (3) extension (20 s at 72 °C); and (4) final elongation step (72 °C for 10 min). Each amplified PCR product was checked in an agarose gel (using 3 µL of the product) along with a negative control to validate the presence of the expected amplified band and eliminate contaminations, before running in a capillary electrophoresis (Figure A1). The final PCR products were separated on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Inc. (ABI), Carlsbad, CA, USA), together with the GeneScanTM-500 LIZ Size Standard (ABI) as internal size standard. Alleles were sized and individuals genotyped using the software STRand 2.4.59.

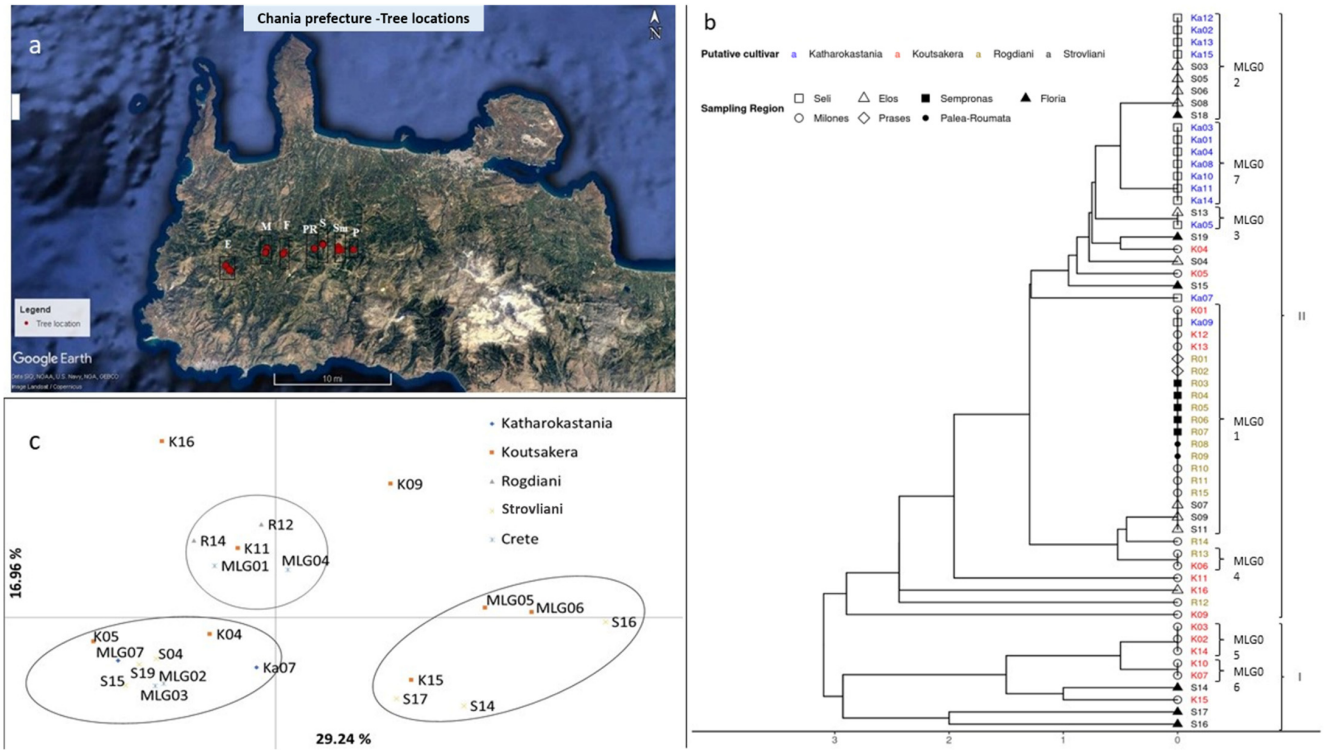

Figure 1. (**a**) Location of the Cretan chestnut sampling sites where the four cultivars were surveyed (Elos (E), Milones (M), Floria (F), Palea Roumata (PR), Prases (P), Selli (S) and Sempronas (Sm)); (**b**) UPGMA tree of the 59 individuals; (**c**) PCoA biplot of the Cretan chestnut trees.

Table 1. Characterisation of the seven loci used to identify the Cretan chestnut varieties. Reproduced from [9–13].

Microsatellites	Reference	Sequence 5′ → 3′	Dye	Size Range	Tm °C
SSRs					
EMCs38	Buck et al. (2003)	TTTCCCTATTTCTAGTTTGTGATG	ROX	214–270	53
		ATGGCGCTTTGGATGAAC	Unlabeled	214–270	53
CsCAT3	Martín et al. (2010)	CACTATTTTATCATGGACGG	FAM	169–275	50
		CGAATTGAGAGTTCATACTC	Unlabeled	169–275	50
CsCAT6	Martín et al. (2010)	AGTGCTCGTGGTCAGTGAG	ROX	158–200	50
		CAACTCTGCATGATAAC	Unlabeled	158–200	50
EST–SSRs					
GOT021	Sullivan et al. (2012)	AGAAAGTTCCAGGGAAAGCA	FAM	93–103	54
		CTTCGTCCCCAGTTGAATGT	Unlabeled	93–103	54
FIR110	Sullivan et al. (2012)	ACTTGCTCGCTTCAACCTTC	TAMRA	166–230	56
		ATTCCTCCTCATCAGGCTCA	Unlabeled	166–230	56
POR042	Martín et al. (2010)	CCACCTGAATCACACGATCT	HEX	111–143	56
		AGTGCATGAATCTCGGGAAG	Unlabeled	111–143	56
WAG004	Martín et al. (2010)	AAAGCAATTCAACTGGGACG	TAMRA	260–288	54
		ACGACACCGTTTGTTCCTTC	Unlabeled	260–288	54

2.3. Data Analysis

The number of alleles (Na), the effective number of alleles (Ne), the observed (Ho) and expected (He) heterozygosity, the number of migrants (Nm), the fixation index (Fst), the Shannon index (I), F-statistics and probability of identity (PI) were processed using GeneAlEx 6.503 [14]. The probability of identity was calculated following [15]. An analysis of molecular variance [16] was also performed using the same software [17]. To examine whether the resulting structure was correlated with geographical distribution, the matrices of the pairwise codom–genotypic genetic distances between all samples and the respective

geographical distances were compared using a Mantel test [18,19]. Allelic richness was calculated using HP-Rare [20].

UPGMA cluster analysis was performed in R 4.1.0, based on relative dissimilarity distance matrix and visualised using the ggtree R package [21]. For the identification of unique genotypes, a multilocus genotype analysis (MLG) for the 59 chestnut trees was performed in R 4.1.0 software (R Core Team, Vienna, Austria) [22] using the poppr 2.9.2 package [23]. As a result, a number of unique genotypes were found and therefore a principal coordinates analysis (PCoA) was performed in GenAlEx.

3. Results

As a result of the multilocus genotype analysis, 22 unique genotypes were identified in the 4 chestnut Cretan cultivars (Table 2). For the 59 chestnut orchard trees studied, 4 different groups of synonymies (genetically identical cultivars with different names), namely, MLG01 to MLG04, were identified. Regarding homonymies (genetically different cultivars with the same name), two homonymy groups were found in both Katharokastania and Strovliani, six in Rogdiani and eight in Koutsakera (Table 2).

Table 2. Multilocus genotype identified among the 59 chestnut accessions of the Cretan chestnut varieties.

MLG	Ka	K	R	S
MLG01	9	1–2–13	1–2–3–4–5–6–7–8–9–10–11–15	7–9–11
MLG02	2–12–13–15			3–5–6–8–18
MLG03	5			13
MLG04		6	13	
MLG05		2–3–14		
MLG06		7–10		
MLG07	1–3–4–8–10–11–14			
MLG08	7			
MLG09		4		
MLG10		5		
MLG11		9		
MLG12		11		
MLG13		15		
MLG14		16		
MLG15			12	
MLG16			14	
MLG17				4
MLG18				14
MLG19				15
MLG20				16
MLG21				17
MLG22				19

In total, 26 alleles were detected in the 7 microsatellite loci. The number of alleles per locus varied between two and seven (average of 3.7 alleles per locus). The most polymorphic locus was GOT021 that presented an observed heterozygosity (Ho) of 0.982 followed by FIR110 and CsCAT6, which presented 0.917 (Table 3). The probability of identity (PI) ranged between 0.615 and 1.000 for WAG004 and CsCAT3, respectively (Table 3).

Table 3. Genetic diversity of simple sequence repeat loci used on the 59 chestnut cultivars of the Cretan chestnut varieties.

	Na	Ho	He	PI	Fis	Fst	Nm	I
				nSSRs				
CsCAT3	7	0.833	0.614	0.239	−0.358	0.140	1.542	1.077
CsCAT6	3	0.917	0.529	0.335	−0.733	0.039	6.142	0.747
EMCS38	5	0.218	0.214	0.652	−0.019	0.065	3.602	0.685
				EST-SSRs				
FIR110	4	0.917	0.570	0.284	−0.607	0.122	1.806	0.955
GOT021	3	0.982	0.516	0.351	−0.904	0.002	161.800	0.756
WAG004	2	0.800	0.459	0.403	−0.743	0.044	5.434	0.615
POR042	2	0.775	0.460	0.401	−0.685	0.045	5.281	0.660
Mean					−0.578	0.065	26.515	
SE					0.112	0.018	22.558	

Na: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PI: probability of identity; Fis: inbreeding coefficient; Fst: fixation index; Nm: number of migrants; I: Shannon index.

The fixation index (Fst = 0.065) shows a relatively low level of differentiation between the cultivars. The average number of migrants ranged between 1.542 for CsCAT3 and 161.8 for GOT021 (Table 3). According to the AMOVA, 73% of the total diversity resides within cultivars and 27% among cultivars (Tables A2 and A3).

A very weak (Pearson's $r = 0.11$), but statistically significant ($p = 0.04$) relationship was found between genetic and geographic distances (Figure A2).

Moreover, the genetic diversity analysis of the separate nSSR and EST-SSR data sets showed that there are no significant differences in the genetic diversity parameters between them (Table A4).

The UPGMA dendrogram distributes the 59 Cretan chestnut individuals across two main clusters at the six allele difference point (Figure 1b). In general, cluster analysis shows a certain Rogdiani group. Moreover, most Katharokastania trees are also clustered together in two MLGs, which differ by only one allele. On the other hand, no clear classification could be made for Koutsakera and especially Strovliani.

In particular, cluster I, represented by a small number of individuals, gathers six putative Koutsakera trees, out of which five are distributed across MLG05 and MLG06 and differ by one allele and one more sample that bears a three allele difference to both MLGs. Cluster I also contains three putative Strovliani trees. Cluster II, which contains the rest of the 59 individuals studied, is represented as follows: (a) 12 Rogdiani individuals, sampled from four different areas, are present as one genotype (MLG01); (b) two other Rogdiani samples separated from the previous by only one allele; (c) an outlier Rogdiani individual "R12", which differed from the main Rogdiani MLG by a five allele difference is also present; (d) Katharokastania individuals, sampled from the Seli region, grouped together in two MLGs (MLG02, MLG07) (MLG02 contains four Katharokastania individuals with only one allele difference from MLG07, which has seven Katharokastania trees); (e) three separated outlier Katharokastania trees, Ka05, Ka07 and Ka09, with a two to three allele difference from the two other Katharokastania MLGs; (f) Strovliani individuals, which show no clear grouping, present a first group of five individuals being genotypically identical to Katharokastania MLG02, a second group of three Strovliani individuals (S07, S09 and S11) present in the putative Rogdiani cluster group (MLG01), while four remaining Strovliani samples (S13, S19, S04 and S15) are scattered with differences of up to three alleles from MLG01 and MLG02. On the other hand, the remaining Koutsakera individuals are dispersed without an obvious pattern across various subgroups of cluster II (Figure 1b).

A PCoA was carried out on the 22 clone-corrected data set. The first two components account for 59.73% of the total variance (Figure 1c). Three different groups could be differentiated. Group I is represented by Rogdiani samples, with two Rogdiani individuals (R12 and R14), in addition to two MLG groups, MLG04 (K06 and R13) and MLG01 (mostly Rogdiani samples) (Table 2; Figure 1c). Group II is dominated by Katharokastania trees,

containing Ka07, MLG07 (Katharokastania individuals), MLG02 (three Katharokastania individuals and five Strovliani individuals), MLG3 (Ka05 and S13), K05 and three Strovliani (S04, S15, S19) (Table 2; Figure 1c). Group III, consisting of Koutsakera and Strovliani cultivars, where Koutsakera dominates with two MLGs (MLG05 and MLG06) and one individual (K15), while Strovliani is represented by three individuals (S14, S16 and S17) (Table 2; Figure 1c).

As far as the sampling regions are concerned, diversity statistics are displayed in Table 4. Allelic richness is reported (results for four gene copies) instead of the number of alleles, in order to account for the unbalanced sampling. The highest allelic richness value occurred in Floria (AR = 2.10) and the lowest in Sempronas (AR = 1.82). An interesting finding was the detection of five private alleles in the Milones region. An AMOVA partitioned the genetic diversity as 22% between regions and 78% within regions (Table 5).

Table 4. Genetic diversity statistics for the seven regions sampled.

Population	N	AR	PA	I	Ho	He	F
Selli	14	1.84	0	0.671	0.796	0.455	−0.699
Milones	20	2.08	5	0.876	0.755	0.540	−0.453
Prases	2	1.86	0	0.594	0.857	0.571	−1.000
Sempronas	5	1.82	0	0.594	0.857	0.476	−1.000
Palea Roumata	2	1.86	0	0.594	0.857	0.619	−1.000
Elos	10	1.97	1	0.744	0.814	0.509	−0.629
Floria	6	2.10	0	0.811	0.619	0.556	−0.257
Overall	59	2.04		0.868	0.776	0.522	−0.479

N: number of samples; AR: allelic richness; PA: private alleles; I: Shannon Index; Ho: observed heterozygosity; He: expected heterozygosity; F: inbreeding coefficient.

Table 5. Analysis of Molecular Variance (AMOVA) for the seven regions sampled.

Source	df	SS	MS	Est. Var.	%	Φ_{PT}	*p*-Value
Among Regions	6	31.747	5.291	0.473	22%		
Within Regions	52	86.355	1.661	1.661	78%	0.222	<0.001
Total	58	118.102		2.134	100%		

4. Discussion

In Crete, chestnut has always been an important component of the natural landscape and traditional agroforestry systems, but it has rarely been assessed genetically before. The present work offers a first genetic assessment of the main Cretan chestnut cultivar germplasm that consists of four widespread cultivars, by using a set of SSR primers. The multilocus genotype analysis showed that in these four Cretan chestnut cultivars there are twenty-two unique genotypes identified. Therefore, each cultivar appears to be a multiclonal variety. This result has also been found regarding other varieties and cultivars, for instance, in Greece [7] and Spain [2].

The results obtained with both nSSR and EST-SSR markers showed high levels of diversity for the Cretan chestnut cultivars, confirming the results obtained in previous studies conducted in European chestnut populations [9,12]. AMOVA revealed that the cultivars are genetically different (presenting some unique allele combinations) but share a common gene pool. These results are in agreement with the relevant literature on chestnut, where most of the variation (~70%) accounted for intracultivar differences [24]. Moreover, the study of Poljak et al. [25] on sweet chestnut wild germplasm and cultivated varieties sampled in central Europe and the western part of the Balkan Peninsula showed that most of the genetic diversity was attributed to the differences between individuals within populations (84.1%), which also supports our findings regarding the partitioning of variation within chestnut cultivars.

The Cretan cultivars exhibit a high level of gene diversity with an observed heterozygosity (Ho = 0.7). These findings are in accordance with the results of Martín et al. [26],

who used nine EST-SSR markers for evaluating Spanish and Italian cultivars (Ho = 0.5 and Ho = 0.6, respectively). On a cultivar basis, both allelic number and observed heterozygosity differ, a finding reflecting the presence of different numbers of genotypes per cultivar. The inbreeding coefficient (F) presents a negative value for all cultivars indicative of a high heterozygosity, and perhaps in relation to cultivar selection for fitness-related traits (such as growth and nut production). Heterozygosity has on numerous occasions been associated with growth and fructification, a phenomenon documented for several years now [27]. In this case, it could be a result attributable to the repeated artificial selection process for higher growth and nut production.

Despite analysing 59 genotypes from a rather restricted geographic area (~2380 km^2), the polymorphism level was notable. The number of alleles per locus was lower compared to the results of other sweet chestnut studies, which nevertheless refer to wider areas. Martín et al. [2] genotyped 100 chestnut trees grown in Andalusia (Huelva and Malaga) with seven microsatellites and detected an average of 5.4 and 7.4 alleles per locus for Huelva and Malaga, respectively. Torello Marinoni et al. [9] genotyped 68 chestnut trees collected in different valleys in northwestern Italy with 10 SSRs and identified 80 alleles with an average of 8 alleles per locus. Moreover, Martín et al. [28] genotyped 239 chestnuts trees in the north, centre and south (Andalusia) of Spain with seven microsatellites and found 13.14 alleles per locus.

The fixation index (Fst) of GOT021 is extremely low compared to the other EST-SSRs (Fst = 0.002), while the number of migrants shows a high value compared to the other EST-SSRs (Nm = 161.8), which indicates that this EST-SSR marker is an outlier marker. Moreover, as a functional marker, it is associated with loci involved in response to drought stress or trait of particular interest [13,29]. This result is in accordance with the findings of numerous relevant studies on *Castanea sativa* and several *Quercus* species, where GOT021 was one of the EST-SSR markers associated with abiotic stress. It was shown, for instance, to be under divergent selection in *Quercus* species [13,30]. Moreover, [13,28,29] did not find strong evidence that this marker is under selection, but they detected private alleles for both tolerant and susceptible *Castanea sativa* trees under drought stress. Additional sampling on a larger area and further analysis (such as a water stress experiment using dedicated genotypes) could provide better insight on GOT021 into adaptation of Cretan chestnut populations.

Cluster analysis revealed a clear connection between some cultivar names as provided by the farmers and the molecular result, while, for some others, no clear correspondence was found. In fact, the Rogdiani cultivar individuals with the same genetic profile sampled from four different regions showed a high degree of confidence regarding cultivar identity. However, it should be pointed out that putative samples from the other three cultivars were also shown to have the same genotype (MLG01). The Katharokastania cultivar samples were almost exclusively grouped into two closely related MLGs (MLG02, MLG07); however, sampling Katharokastania individuals from only one region (Seli), in addition to the presence of some outlier Katharokastania individuals within other cultivar groups, blurs the assertion for cultivar identity. Regarding the Koutsakera and Strovliani cultivars, no clear molecular identification was possible, as individuals from both cultivars are dispersed across various subgroups. Nevertheless, it is worth noting that a subsample of putative Koutsakera samples was grouped into a unique cluster identified in the dendrogram (cluster I) as well as in the PCoA.

The PCoA results are in accordance with the cluster analysis results, revealing the Rogdiani and Katharokastania cultivar identity (the latter with less confidence, as some individuals from other cultivars were grouped within the Katharokastania group). On the other hand, no clear differentiation for the Koutsakera and Strovliani cultivars was seen. As the chestnut cultivars studied are classified as widespread traditional varieties in Crete, it is not uncommon to have cases where misnaming of some trees can occur between farmers, especially if those cultivars share some fruit characteristics.

There were 18 homonym and 4 synonym groups distinguished in this study. As the original cultivar identification by name was given during sampling by the respective owners of the orchards where these traditional varieties are present, there appears to be some confusion in practice as to cultivar identity. The same was observed in Spain; [2] reported two homonym and four synonym groups found among the Spanish cultivars studied. Given the amount of genetic variation found and the cases of homonyms and synonyms, the sample size per nominal cultivar to assess intracultivar variation should be increased in future studies. The four cultivars could be distinguished in two groups. Katharokastania and Rogdiani are relatively low-number multilocus genotype cultivars (five and four MLGs, respectively), while Koutsakera and Strovliani can be considered as high-number multilocus genotype cultivars (10 and 9 MLGs, respectively). In terms of chestnut crop uniformity, the nuts produced from Katharokastania and Rogdiani are expected to be more uniform given their low MLG number. On the other hand, Koutsakera and Strovliani present low uniformity which reduces the market value of their chestnut crop. In fact, Koutsakera, a low uniformity variety, has the highest standard deviation in nut area, while Katharokastania, a high uniformity variety, has the least (El Chami et al., unpublished results). Nevertheless, under strong environmental pressure (for example, under climatic change), more MLGs within a cultivar should offer better long-term orchard stability.

No relationship was found between genetic and geographic distances, similar to the results reported for Spanish cultivars [2,24]. Due to isolation by distance, in typical natural populations, genetic and spatial distances are usually positively correlated. On the other hand, an orchard where the placement of cultivars on the ground is an anthropogenic exercise explains the absence of a relation between genetic and geographic distance. This finding has also been observed in a similar study in Greece [10]. This result also indicates that the chestnut orchards in the Chania region are either planted or grafted onto wild rootstock; the presence of any remnants of old-growth natural chestnut forest in the studied region is not supported by our results. Groups of trees from all regions exhibited similar levels of genetic diversity. Surprisingly, five out of the six private alleles detected in this study originated in Milones, which might reflect a greater significance of this area as a centre of traditional chestnut cultivar diversity. However, some caution should be exercised in this interpretation, due to the unbalanced sampling design. The fact that AMOVA partitioned most of the genetic variation within regions (78% within regions, 22% between regions) points towards the employment of similar cultivation and/or domestication practices in the past centuries throughout the investigated area.

5. Conclusions

The null hypothesis of no significant genetic diversity within a cultivar denomination is rejected. The considerable genetic variation found indicates that the Cretan chestnut germplasm may form a gene pool that warrants further investigation. Future research could employ more intense sampling schemes and/or a holistic approach to exploring this important germplasm by integrating the study of flowering characteristics [10] and chemical quality attributes [31] in addition to detailed genetic evaluation. Such analyses will complement the assessment of the performance and real value of this genetic heritage, prior to undertaking actions for its conservation and sustainable utilisation in prebreeding and breeding programmes.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. WGS84 GPS coordinates for the surveyed Cretan chestnut trees representing local varieties.

Tree Code	Cultivar	Region	Lat.	Long.
Ka01	Katharokastania	P. ROUMATA	35.38622	23.79691
Ka02	Katharokastania	P. ROUMATA	35.38620	23.79706
Ka03	Katharokastania	P. ROUMATA	35.38627	23.79714
Ka04	Katharokastania	P. ROUMATA	35.38613	23.79702
Ka05	Katharokastania	P. ROUMATA	35.38606	23.79695
Ka07	Katharokastania	P. ROUMATA	35.38583	23.79660
Ka08	Katharokastania	P. ROUMATA	35.38578	23.79645
Ka09	Katharokastania	P. ROUMATA	35.38642	23.79558
Ka10	Katharokastania	P. ROUMATA	35.38645	23.79558
Ka11	Katharokastania	P. ROUMATA	35.38653	23.79565
Ka12	Katharokastania	P. ROUMATA	35.38747	23.79630
Ka13	Katharokastania	P. ROUMATA	35.38752	23.79636
Ka14	Katharokastania	P. ROUMATA	35.38747	23.79618
Ka15	Katharokastania	P. ROUMATA	35.38744	23.79652
K01	Koutsakeri	MYLONES	35.37575	23.70544
K02	Koutsakeri	MYLONES	35.37583	23.70548
K03	Koutsakeri	MYLONES	35.37592	23.70554
K04	Koutsakeri	MYLONES	35.37586	23.70563
K05	Koutsakeri	MYLONES	35.37599	23.70560
K06	Koutsakeri	MYLONES	35.37605	23.70570
K07	Koutsakeri	MYLONES	35.37595	23.70538
K09	Koutsakeri	MYLONES	35.38110	23.70666
K10	Koutsakeri	MYLONES	35.38123	23.70668
K11	Koutsakeri	MYLONES	35.38133	23.70668
K12	Koutsakeri	MYLONES	35.38142	23.70667
K13	Koutsakeri	MYLONES	35.38149	23.70667
K14	Koutsakeri	MYLONES	35.37601	23.70529
K15	Koutsakeri	MYLONES	35.37586	23.70521
K16	Koutsakeri	ELOS	35.35276	23.65008
R01	Rogdiani	PRASSES	35.37936	23.84548
R02	Rogdiani	PRASSES	35.37963	23.84559
R03	Rogdiani	SEMPRONAS	35.37826	23.82102
R04	Rogdiani	SEMPRONAS	35.37933	23.82069
R05	Rogdiani	SEMPRONAS	35.38317	23.82118
R06	Rogdiani	SEMPRONAS	35.38308	23.82122

Table A1. *Cont.*

Tree Code	Cultivar	Region	Lat.	Long.
R07	Rogdiani	SELLI	35.37867	23.82611
R08	Rogdiani	P. ROUMATA	35.38079	23.78319
R09	Rogdiani	P. ROUMATA	35.38079	23.78308
R10	Rogdiani	MYLONES	35.37541	23.70487
R11	Rogdiani	MYLONES	35.37536	23.70486
R12	Rogdiani	MYLONES	35.37529	23.70483
R13	Rogdiani	MYLONES	35.37610	23.70535
R14	Rogdiani	MYLONES	35.37632	23.70527
R15	Rogdiani	MYLONES	35.37653	23.70510
S03	Strovliani	ELOS	35.35883	23.64221
S04	Strovliani	ELOS	35.35874	23.64238
S05	Strovliani	ELOS	35.35876	23.64248
S06	Strovliani	ELOS	35.35874	23.64259
S07	Strovliani	ELOS	35.35196	23.65058
S08	Strovliani	ELOS	35.35213	23.65052
S09	Strovliani	ELOS	35.35226	23.65044
S11	Strovliani	ELOS	35.35319	23.64848
S13	Strovliani	ELOS	35.35334	23.64834
S14	Strovliani	FLORIA	35.37624	23.73497
S15	Strovliani	FLORIA	35.37624	23.73487
S16	Strovliani	FLORIA	35.37392	23.73303
S17	Strovliani	FLORIA	35.37391	23.73321
S18	Strovliani	FLORIA	35.37392	23.73331
S19	Strovliani	FLORIA	35.37402	23.73326

Appendix B

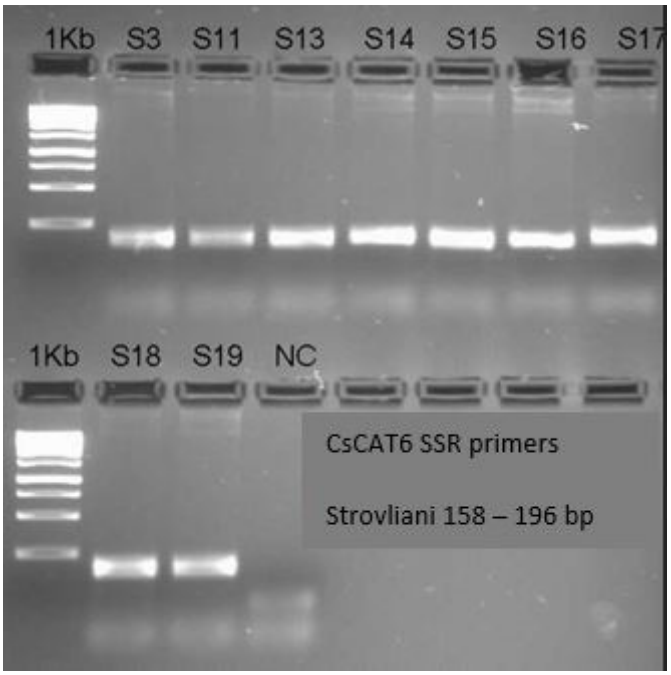

Figure A1. Agarose gel picture of Strovliani chestnut variety samples using the CsCAT6 SSR primer.

Appendix C

Table A2. Results of the Analysis of Molecular Variance regarding of the Cretan chestnut varieties studied.

Source	d.f.	S.S.	M.S.	Est. Var.	%
Among Cultivars	3	31.164	10.388	0.597	27%
Within Cultivars	55	86.938	1.581	1.581	73%
Total	58	118.102		2.178	100%

Table A3. PhiPT estimation based on the Analysis of Molecular Variance of the Cretan chestnut varieties studied.

Statistic	Value	*p* Value
PhiPT	0.274	<0.001

Appendix D

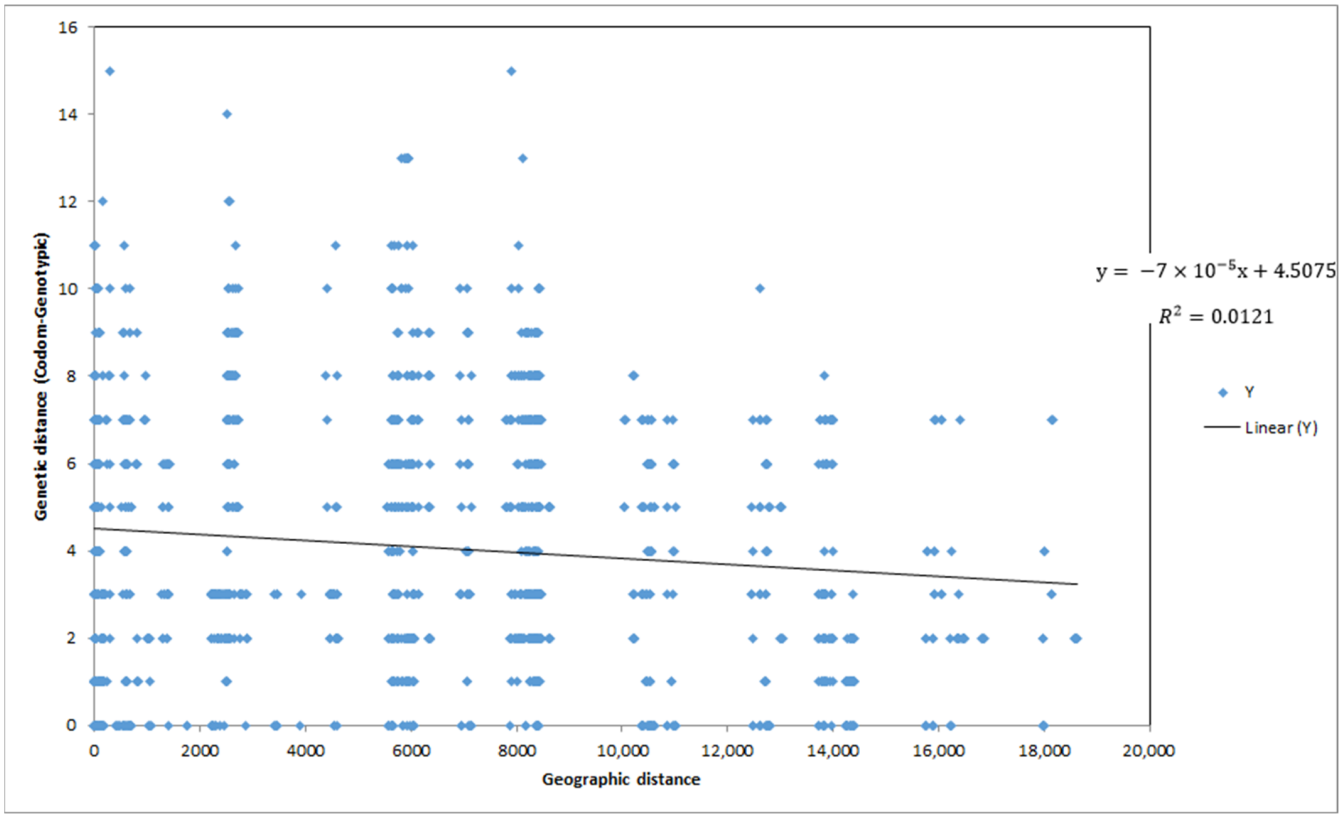

Figure A2. Correlation between genetic data with geographical data of the studied Cretan chestnut cultivars.

Appendix E

Table A4. Diversity parameters for the nSSRs and EST-SSRs of the studied Cretan chestnut cultivars.

	(I)	Ho	He	Na	AMOVA	F	PCoA
nSSR	0.761	0.66	0.45	3.08	72% within 28% among	−0.37	66.13% Axe1 and Axe2
ESTSSR	0.745	0.87	0.5	2.44	73% within 27% among	−0.73	53.77% Axe1 and Axe2
t-test	0.879	0.09	0.41	0.13		0.055	

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