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# Genetic Diversity of *Castanea sativa* Mill. Accessions from the Tuscan-Emilian Apennines and Emilia Romagna Region (Italy)

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**Abstract:** This work investigated the genetic diversity of 134 *Castanea sativa* Mill. accessions present in the Italian region of Emilia-Romagna. Samples were taken from three collection fields (Granaglione, Zocca and Paloneta) in the Tuscan-Emilian Apennines. The accessions were analyzed by using 16 microsatellite markers (SSR). Genetic distances among accessions, calculated through the DICE coefficient, were used to construct an UPGMA cluster analysis. One major genotype (named “Marroni”) was identified across the three investigated collection fields; this variety corresponds to a sweet chestnut cultivar that has been propagated and widely diffused in the Emilia-Romagna region. Other genotypes were represented by different varieties of Italian chestnuts. The results of this study will be used to define and share guidelines for the characterization and varietal certification of the chestnut varieties in the Emilia-Romagna region.

**Keywords:** chestnut; genetic diversity; local germplasm; SSR; cluster analysis

## 1. Introduction

To date, the natural distribution area of the European chestnut (*Castanea sativa* Mill.) mainly includes southern Europe and southwestern Asia. In particular, the European distribution area extends from the northwestern part of the Iberian Peninsula to Caucasia and the Caspian Sea [1].

According to palaeobotanic data, the current biodiversity of the chestnut tree originates from glacial refugia located in Transcaucasia and in the Italian and Iberian peninsulas, where chestnut trees probably found a favorable habitat. During the Holocene, chestnut trees spread to the surrounding areas as a result of post-glacial climate conditions and human activities [2].

The first unambiguous evidence of chestnut cultivation was reported in the Middle East and Greece and dates back to about 4000 B.C., although chestnut use was reported during the Neolithic (6000 BP) [3]. Subsequently, in the Greek and pre-Christian world, chestnut tree cultivation was a minor activity.

The role of the chestnut changed at the beginning of the Christian era, when the versatility of this tree was better understood. In Italy, and thereafter in Europe, chestnut cultivation might have been introduced by the Romans, although there is no clear evidence of systematic tree planting in the Italian territory [4].

During the Middle Ages, the cultivation of chestnut in the Italian Apennines intensified thanks to Matilde di Canossa around the year 1110 A.D. To render the territory self-sufficient, she strongly

encouraged the cultivation of chestnut in this region, which is why many old and monumental trees in this area are named “Matildici” [4].

The increase of chestnut cultivation led to the birth of the idea of tree/fruit selection: productivity, size and flavor of the fruit started to be taken into account [5]. An example of such development is given by the Marroni genotype.

In the 16th century, in a region between Tuscany and Emilia-Romagna, a cultivar called ‘Marrone Fiorentino’ was selected and propagated throughout different regions of central and northern Italy [5–7].

The Marroni genotype (or sweet chestnut) was selected for its excellent characteristics: (i) weight of the fruit above average (maximum 70 fruits per kg); (ii) one to three fruits per burr; (iii) monoembryonic nuts; (iv) epicarp of bright light color, marked with accentuated grooves of darker coloring; (v) thin and easy-to-remove epispem (cuticle), not deep in the cotyledons; (vi) floury paste, sugary, consistent, resistant to cooking without breaking up [7]. Another feature among the Marroni accessions is that they are androsterile.

The genetic uniformity among Marroni group accessions is the result of clonal propagation carried out by growers to maintain the desired characteristics [8,9]. Subsequently, the Marroni genotype was planted in various areas, where it was given different names, such as Marrone di Castel del Rio, Marrone di Zocca, Marrone Buono of Marradi, Marrone Biondo di Monghidoro and others [5–7,10].

Other than the Marroni genotype, in the Tuscan-Emilian Apennines, the other dominant varieties of chestnut, which are mainly used for the production of flour and other derivatives, are the following: ‘Carpinese’ or ‘Carrarese’, ‘Pastanese’, ‘Pistolese’, ‘Piusela’, ‘Ceppa’ and ‘Loiola’ [5,11,12]. These chestnut cultivars are characterized by variable fruit weight (each cultivar presenting a specific weight range), polyembryonic nuts with an adherent and intrusive epispem and lower fruit sweetness compared to the Marroni group [7,12].

To date, considering Italy as a whole, chestnut trees are mainly present in six regions of the country (Campania, Lazio, Tuscany, Emilia-Romagna, Piedmont, Veneto). Italian varieties are characterized by a wide genetic variability resulting from a tradition of multiplying the varieties by seed. This tradition contributed both to a high number of native ecotypes throughout the country and to the subsequent selection of cultivars that, over time, have adapted to different areas. Each has specific characteristics that are regulated by the PGI (Protected Geographical Indication) issued by the European Union [13,14]. In particular, Marrone of ‘Castel del Rio’ has been awarded PGI certification, being one of the most valuable and known chestnut cultivars in Italy and abroad, originating from the Emilia Romagna region.

The highest number of varieties is cultivated in Tuscany (26.9%), followed by Piedmont (15.2%), Campania (12.8%), Emilia Romagna (8.8%) and Calabria (7.5%) [14].

Nowadays, there are many challenges that threaten chestnut production in the Tuscan-Emilian Apennines, e.g., the diffusion of pathogens and pests such as the *Gnomoniopsis ascoe* fungus and the Oriental chestnut gall wasp, *Dryocosmus kuriphilus* [15]. In addition, there are socio-economic problems related to the market and to a rapidly changing environment [16]. These elements encourage the in-depth study of the chestnut tree and the enlargement of local germplasms to preserve the existing biodiversity and eventually identify desirable traits, such as resistance to pests or features that could be potentially useful to the Italian chestnut industry.

The identification of redundant accessions (identical genetic profile but with a different name) represents a fundamental preliminary step to undertake a genetic characterization of the germplasm, since most of the accessions have been found in the fields and initially identified with their local names [17,18].

In such cases, it is necessary to support a further phenotypic analysis, using pomological charts to verify the presence of a true state of synonymy, if known, or to identify different phenotypes probably due to point genetic mutations, structural genome changes or epigenetics [18].

Molecular markers, such as Single Sequence Repeats (SSRs) or microsatellites, can support pomological analyses and have been used for genetic diversity analysis (and for structure analysis)

in several fruit tree species (in grapevine [18]; in apple [19,20]; in hazelnut [21]; in pear [22,23]). The related datasets have provided a useful support for varietal identification. The same approach can be used for the analysis of the genetic diversity of chestnuts as well. This approach was used to characterize germplasm collections [8,9,24] and to describe the existing relationships among Italian and European varieties [25–29]. The use of SSRs allowed the identification and characterization of traditional varieties from southern Spain [8,30]. These studies could be used as a model in order to extend the analysis to other regional germplasms in Italy and Europe [24,27,29], to characterize the collections and to provide tools for varietal certification.

Currently, the characterization of chestnut biodiversity in Emilia Romagna has been mainly performed by means of pomological and morphological analyses. The genetic information available is still limited. These morphological descriptions are available in the regional repertoire of the varieties at risk of genetic erosion.

Therefore, the main objectives of this study are: a) to describe the biodiversity of the existing ecotypes and to preserve the existing chestnut heritage from further genetic erosion; b) to provide a genetic database of the main cultivars in Emilia Romagna for traceability and conservation purposes.

## 2. Materials and Methods

### 2.1. The Origin of the Biological Material

A panel of 134 accessions were collected in the area of the Tuscan-Emilian Apennines. In particular the sampling was carried out in three collections fields: Parco Didattico Sperimentale del Castagno di Granaglione, the Collection of Zocca and Paloneta (created in Emilia Romagna by the University of Florence). These fields are characterized by the presence of several grafted replicates of varieties known only at phenotypic level so far (Figure S1; Table S1).

### 2.2. Molecular and Genetic Diversity Analyses

For each accession, genomic DNA was extracted from 50 mg of young freeze-dried leaves following the standard CTAB protocol [31]. Genomic DNA was quantified using the Nanodrop<sup>TM</sup> ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 10 ng/ $\mu$ L.

The PCR reactions were performed with the thermal cycler 2700 GeneAmp PCR System (ABI Prism) and carried out with 9  $\mu$ L of master mix and 1  $\mu$ L of DNA template. The PCR reactions followed this amplification protocol: an initial denaturation step of 10 min at 95 °C, followed by 35 cycles for 30'' at 95 °C, and 30'' at specific annealing temperature (Table S2), and 30'' at 72 °C, with a final extension step of 7' at 72 °C.

Preliminary phases of genetic characterization focused on the estimation of genetic diversity and on the determination of genetic relationships within the studied germplasm. Molecular markers (SSR) allowed to create a fingerprint for each single variety.

The microsatellites used were selected by the series (CsCAT and EMCs) and OAL elaborated on the chestnut [32–34] and QrZAG developed from *Quercus robur* [35].

In order to characterize regional varieties, the samples were amplified by 16 pairs of labeled primers which were found to be the most polymorphic. The primers were used by multiplex set according to Pereira-Lorenzo et al. [27] (Table S2). In order to estimate the size of DNA fragments, the samples were aligned with the European dataset [27].

### 2.3. Genetic and Cluster Analysis

The number of alleles per locus ( $k$ ), the expected ( $H_e$ ) and the observed heterozygosities ( $H_o$ ) and the polymorphism information content (PIC) of the unique genotypes were estimated using the CERVUS Software Version 3.0.3 [36]. A PIC value greater than 0.7 was considered to be highly polymorphic and informative for a certain locus. A Parentage analysis on unique diploid genotypes

with the CERVUS software [36,37] was carried out. Two criteria were considered to establish parental relationships: a LOD confidence interval and the Delta value with a threshold of 95%.

Using all the obtained data, a cluster analysis was carried out with the construction of the dendrogram relative to genetic distances, elaborated using the Unweighted Pair-Group Method (UPGMA). The genetic distance between the cultivars was calculated using the DICE coefficient [38] with the SimQual NTSYSpc 2.0 [39].

To have further confirmation on the genetic similarities previously observed with the cluster analysis, the R software (Project for Statistical Computing, version 3.2.2, Copyright (C) 1989, 1991 Free Software Foundation, Inc. 59 Temple Place, Suite 330, Boston, MA 02111-1307 USA) was used to perform the principal component analysis (PCA) on the 21 unique genotypes identified.

### 3. Results

The 16 selected molecular markers allowed the analysis of the genetic diversity and provided useful support for the direct analysis of varietal identification. In general, allele frequencies were not uniformly distributed within the investigated loci. The unique genotypes identified showed frequencies ranging from very low (as for the EMCs2 locus) to very high for the CSCAT3 locus, with 3 and 16 alleles, respectively. The 16 SSRs used in this study revealed a total of 132 alleles, with an average of 8.2 alleles per locus. Comparing the size of the DNA fragment with the chestnut EU dataset [27], 6 unique alleles were found in 21 unique genotypes: CSCAT 16-128; CSCAT3-227 and 257; CSCAT1-179, QrZAG96-163 and EMCs38-234 (in bold in Table S3).

The absence of amplification of EMCs38 on one genotype ('Madonna, Table S3) may be due to the presence of null alleles. For this reason, the 'Madonna' genotype was not considered in the heterozygosity analysis, which was carried out with 20 unique varieties.

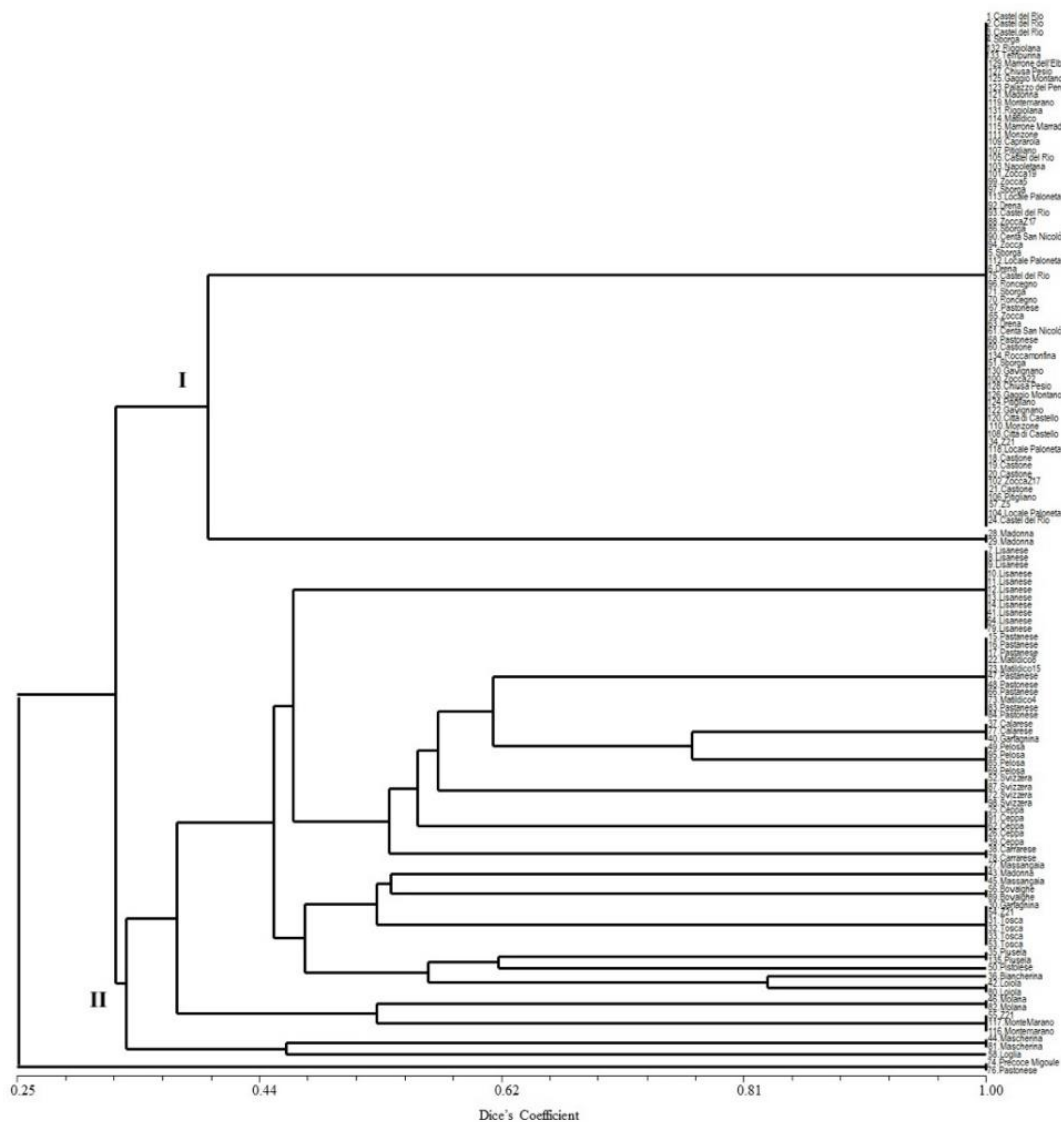
CSCAT3 and EMCs38 with a PIC value around 0.885 and 0.801 appeared to be the highest informative loci. Conversely, OAL and EMCs15 with a PIC value = 0.300 and 0.473 respectively are the least informative.

The high value of expected heterozygosity directly reflects the high level of genetic diversity present in chestnut trees derived from cross-pollination: the value of observed heterozygosity ranged between 0.350 for OAL to 1 for CsCAT14, whereas the expected heterozygosity ranged between 0.319 for OAL to 0.917 for CsCAT3 (Table 1).

**Table 1.** The number of individuals (N), the number of alleles (k), the observed (Ho) and expected (He) heterozygosity and the polymorphic information content (PIC) are reported for each SSR locus in *C. sativa* accessions.

Locus	k	N	Ho	He	PIC
CsCAT41	10	20	0.750	0.763	0.710
CsCAT16	9	20	0.900	0.840	0.795
CsCAT6	8	20	0.900	0.823	0.773
CsCAT1	9	20	0.750	0.710	0.662
CsCAT3	16	20	0.850	0.917	0.885
QrZAG96	6	20	0.750	0.731	0.674
EMCs15	6	20	0.600	0.521	0.473
EMCs38	13	20	0.750	0.842	0.801
EMCs2	3	20	0.750	0.668	0.577
EMCs22	7	20	0.750	0.760	0.703
CsCAT2	11	20	0.800	0.831	0.794
CsCAT17	8	20	0.750	0.827	0.780
CsCAT14	7	20	1.000	0.787	0.730
CsCAT15	5	20	0.650	0.573	0.499
CsCAT8	9	20	0.900	0.827	0.779
OAL	5	20	0.350	0.319	0.300

The dendrogram derived from the analysis of the molecular profiles allowed the identification of the similarities and / or identity among the studied samples (134 accessions in total; Table S1), highlighting, in particular, the distinction between the varieties of sweet chestnut (Cluster 1) and chestnut (Cluster 2; Figure 1).



**Figure 1.** Dendrogram based on 16 SSRs using DICE coefficient demonstrating genetic characterization and relationship of 134 chestnut accessions from the Tuscan-Emilian Apennines, divided in two clusters.

Cluster 1 included 66 accessions of sweet chestnut with a uniform molecular profile even if the samples had been classified with different names, confirming synonymy among the Marroni group: ‘Caprarola’, ‘Castel del Rio’, ‘Castione’, ‘Centa di S. Nicolò’, ‘Chiusa Pesio’, ‘Città di Castello’, ‘Drena’, ‘Gaggio Montano’, ‘Gavignano’, ‘Locale di Paloneta’, ‘Marron Buono di Marradi’, ‘Marrone dell’Isola d’Elba’, ‘Montemarano’, ‘Monzone’, ‘Napoletana’, ‘Palazzo del Pero’, ‘Pitigliano’, ‘Roccamonfina’, ‘Riggolana’, ‘Roncegno’, ‘Sborgà’, ‘Tempurina’ and ‘Zocca’. Our results therefore indicated that the Marroni group is represented by a single genotype named ‘Marrone Fiorentino’ described in the EU chestnut database [27].

This cluster also included accessions of Marroni called ‘Pastonese’, which should not be confused with the ‘Pastanese’ chestnut variety, as well as a Marroni accession called ‘Madonna’ that differs from

the ‘Madonna’ chestnut variety. This was also observed for the accession known as ‘Montemarano’. In addition, an old ‘Matildico’ tree was found in the Marroni group.

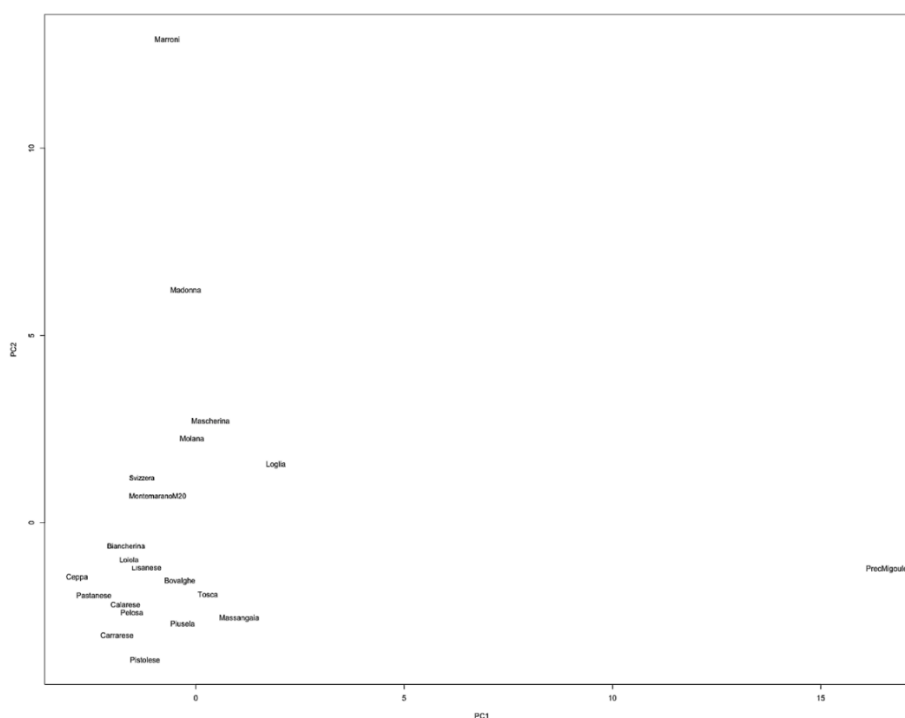
Conversely, Cluster 2 showed higher variability, forming numerous sub-clusters. 20 different chestnut genotypes were identified in a total of 68 accessions (Figure 1; Table S3). The dendrogram showed solid sub-clusters of accessions labelled: ‘Lisanese’, ‘Pastonese’, ‘Mascherina’, ‘Calarese’, ‘Pelosa’, ‘Svizzera’, ‘Ceppa’, ‘Carrasere’, ‘Bovalghe’, ‘Massangaia’, ‘Piusela’, ‘Loglia’, ‘Molana’ and ‘Tosca’. This indicated a good propagation of the chestnut varieties in the Tuscan-Emilian Apennines area (Figure 1). These genotypes were separated in the dendrogram from the chestnut cultivars from southern Italy, such as ‘Montemarano’ (mainly cultivated in the Campania region).

As shown in Figure 1, the ‘Precoce Migoule’ variety, a hybrid cultivar deriving from *Castanea sativa* × *Castanea crenata* [40], turned out to be very distant from the local chestnut cultivars, as most of the informative loci have different alleles (dataset in Table S3).

The dendrogram for Cluster 2 also revealed the presence of synonymous accessions (identical SSR profile but different cultivar name) such as ‘Garfagnina’ and ‘Tosca’. Furthermore, the ‘Pastanese’ accessions were grouped together with ‘Pastonese’ accessions and several ‘Matildico’ trees (4-8-15).

Occasional misnomers have been found by SSR analyses, such as an accession called ‘Garfagnina’ in the group of the ‘Carrarese’ cultivar, the accession named ‘Z21’ in the ‘Tosca’ genotype group and an accession named ‘Pastonese’ with an allelic profile identical to ‘Precoce Migoule’.

In conclusion, the 134 accessions analyzed showed 21 different genotypes representative of the Emilia-Romagna biodiversity (Table S3), with a clear separation between the Marroni group accessions (Cluster 1) and all chestnut varieties from central and southern Italy (Cluster 2). In addition, a Principal Coordinate Analysis (PCoA) on the 21 previously identified unique varieties was conducted with the R software. Figure 2 shows that the ‘Precoce Migoule’ varieties, a hybrid cultivar, differ considerably from the varieties present in the Tuscan-Emilian Apennines, which formed a small cluster. Furthermore, the ‘Madonna’ genotypes and Marroni group were found to be more similar to each other but separated from all the other chestnut varieties.



**Figure 2.** Principal Coordinate Analysis (PCoA) of the 21 chestnut unique genotypes based on the 16 SSR data. The first component (PC1) explains 15% of the variation and the second component (PC2) 11%.

A parentage analysis was carried out by CERVUS and was performed excluding the locus EMCs38, which may be present null alleles. The parentage analysis did not reveal possible parental relationships (data not shown).

#### 4. Discussion

In this study, we performed the molecular characterization of a collection of 134 grafted chestnut and sweet chestnut (Marroni group) accessions from different collections in the Emilia-Romagna region, which corresponded to 21 representative varieties. The relatively high number of accessions of the dataset (with varieties that are well distributed in the regional territory and also include commercially used varieties) provided a good overview of the distribution of grafted chestnut varieties in the region. The set of SSRs used in this study was chosen mainly on the basis of their distribution throughout the chestnut genome, in order to reach a high value of genomic coverage to estimate the population's genetic diversity. This marker set was also used in the genetic diversity study of Spanish chestnut germplasm described by Pereira Lorenzo et al. [27]. A work by Urrestarazu et al. [41] studied the variations in the results of genetic diversity analysis in relation to the number of markers used. This work identified that 15-16 is the ideal number of markers for this type of analysis and asserted that a higher number of markers does not positively influence the statistical stability of the results.

The present study was based on a molecular analysis using 16 specific SSRs. Their high variability made it possible to amplify and visualize numerous alleles (the mean of 8.2 alleles). The high degree of polymorphism and high discriminating power among the analyzed samples was expected for a cross-pollination species, such as *C. sativa*.

The presence of unique alleles was found in five SSRs tested. This evidences a relevant genetic diversity among the *C. sativa* species due to the high discriminant power of the molecular marker set used.

Our molecular marker set was picked with the intent of creating an effective varietal identification tool for future use, as many other crops have.

In particular, the CsCAT3 primer was found to be one of the most discriminating loci (PIC=0.885), as already confirmed by other studies [17,42,43]. These markers should be checked as a first step to identify varieties in Piedmont with the EU database [27].

Conversely, the EMCs series of loci, being trinucleotide SSRs, mutate at a lower rate than dinucleotide SSRs (CSCAT series), resulting in lower polymorphism [25], as was the case for EMCs15 (PIC=0.473). In addition, the OAL marker [34] presented the lowest capacity for discrimination (PIC=0.300), further emphasizing the lower values of heterozygosity ( $H_o = 0.350$ ;  $H_e = 0.319$ ).

The cluster analysis showed an overall high genetic diversity, which demonstrated the importance of characterizing the chestnut trees present in this territory. The traditional cultivars are frequently called according to geographic origin, ripening period and traits of the nut, creating difficulties in their classification [30,34]. For example, the name 'Pelosa' is a cultivar known in Emilia-Romagna and also in Piedmont for the big nut size and the presence of hairiness on the epicarp of the nut, as suggested by its name [44].

The study evidences that each area presented its own specific chestnut genotype (represented by Cluster 2): 'Piusela' varieties in the Reggio-Emilia area, 'Pelosa', 'Lisanese' and 'Pastanese' in the Tuscany Apennines and 'Montemarano' in Campania [8,12,40].

This was also confirmed by the Principal Component Analysis in which chestnut varieties from the Tuscan-Emilian Apennines were found to be close to each other and separated from the varieties of southern Italy and from the 'Precoce Migoule' hybrid cultivar.

Furthermore, the 'Pastanese' cultivar and 'Matildico' trees were found to belong to the same genotype which is known for the production of high-quality flour. It is at least arguable, therefore, that the 'Matildici' cultivars could be the very cultivars planted by 'Matilde of Canossa' in the Middle Ages [7,12,45,46].

The presence of ancient trees and known varieties in the same cluster had already been described in Italy and Spain [28] and in Switzerland [24].

On the contrary, the molecular results from sweet chestnut trees (Marroni group, Cluster 1) showed a uniform profile sharing the same allelic profile as a result of clonal propagation. This is because they were selected by growers to maintain the desired characteristics, such as high quality monoembryonic nuts with high nut weight and thin episperm (cuticle) with a floury and sweet taste [7]. These results are further confirmed by pomological characterization evidencing a high rate of homogeneity in the Marroni group [5–7]. The selection and cultivation of these clones led to the spread of the Marroni group in distinct geographical areas. Later on, environmental factors affected the nuts' morphological aspects [6], leading to different denominations such as 'Marrone di Castel del Rio', 'M. di Zocca', 'Centa di San Nicolò', 'Roncegno', 'Drena', 'Marrone di Gaggio Montano', which are synonyms of the Marroni Fiorentino described in the EU chestnut database [27,28]. Further Marroni groups with the same molecular profile, such as cv. 'Marrone di Cuneo', 'Marrone di Combai' and 'Chiusa Pesio', were also described in Piedmont [8,9,44] and showed a different genetic profile compared to the 'Marrone di Cuneo' (genetic synonym of 'Marrone Gambarogno') found in Switzerland [24].

Summarizing, the results obtained from Tuscan-Emilian Apennines varieties confirmed the close relationship between the diffusion of the genotypes and local population. Where farmers focus on clonal propagation for production purposes, such as for the Marroni group (Cluster 1), the genetic diversity of the crop is reduced. By contrast, the chestnut group (Cluster 2) featured a higher genetic diversity between distinct gene pools due to the selection of trees originated by seeds and propagated by grafting among a broad genetic base which led to a reduction of differences between wild and cultivated chestnut trees [28].

Finally, this research work points out the importance of *ex situ* collections so as to provide plant material for breeding programs and for nursery propagation. The availability of the molecular profile for several varieties will support the varietal classification activity, which is currently more difficult, as many genotypes were cultivated in different regions with different denominations.

## 5. Conclusions

In conclusion, the performed molecular characterization allowed the correct identification of the varieties mainly cultivated in the area of the Tuscan-Emilian Apennines. The identification of synonymous accessions emphasized the importance of verifying collections of germplasm with powerful tools such as molecular markers. These tools are fundamental to avoid both redundancy and possible issues of varietal certification for propagation in nurseries.

Furthermore, this research promotes the diffusion of ecotypes to promote the preservation of chestnut biodiversity with the inclusion of varieties at risk of genetic erosion. The involvement of local farmers as project partners increased their awareness of underlying matters and their availability to host and guard plants at risk of genetic erosion. Genotypes at risk, e.g., the Marroni group, must be reintroduced taking into account soil and climate characteristics.

This research also analyzed the genetic diversity with the aim of enriching collection fields in the Emilia-Romagna region through identified unique varieties. The results confirm that the Italian chestnut germplasm is an important source of genetic biodiversity and contributes to the preservation and enhancement of the entire chestnut genetic heritage.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/9/1319/s1>, Figure S1: Maps of the three collection fields analyzed in Emilia-Romagna region, Italy: A) Paloneta (FA); B) Zocca (MO) and C) Parco Didattico Sperimentale del Castagno di Granaglione (BO), Table S1: List of the 134 varieties sampled in tree collection camps of the Emilia Romagna, Table S2: SSRs markers used in amplifications (Pereira-Lorenzo et al., 2017). LG: Linkage group; FAM; VIC; NED; PET (fluorochromes used in PCR analysis), Table S3: Allelic profiles of 21 varieties (prime name, synonyms and number of accessions analyzed) from the Emilia Romagna region for 16 SSR (-1 for missing value).

**Author Contributions:** Methodology, S.A., S.P.-L., L.D.; Software, S.A., A.M.R.C., L.D.; Resources, S.A., M.K., D.A., L.D.; Data Curation, S.A., M.K., D.A., L.D., A.M.R.C.; Writing—Original Draft Preparation, S.A.;



Writing—Review & Editing, L.D. and S.P.-L.; Supervision, L.D.; Project Administration, L.D.; Funding Acquisition, L.D.". All authors have read and agreed to the published version of the manuscript.

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

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