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Genetic characterization of Italian and Spanish wild and domesticated chestnut trees

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Abstract

The only native species of the *Castanea* genus in Europe is C*astanea sativa* Mill., a widespread and important multipurpose tree species in the Mediterranean area that provides fruit, wood and shelter for hives. With the aim of expanding the knowledge of the genetic variability of the chestnut species (wild trees and varieties) and promoting the traceability of local products, an analysis based on 16 SSRs was carried out on 630 single trees from Italy and Spain. 319 unique genotypes were identified. A Bayesian approach combined with the Markov Chain Monte Carlo (MCMC) simulation method revealed the existence of two genetically distinct groups of chestnuts: Cluster 1 (Spain) and Cluster 2 (Italy), with a clear separation between the cultivars from (northern and southern) Spain and from Italy. The results also confirmed a common genetic structure between chestnut populations from southern Spain and southern Italy, which is the result of historical events and long-term

human impact. The results showed no genetic differentiation between chestnut cultivars (grafted trees) and wild chestnut trees, probably as a consequence of the proximity of orchards and natural populations, which resulted in a gene flow between them.

Keyword: *Castanea sativa* Mill., microsatellite markers (SSRs), Genetic diversity, Germplasm conservation, Structure analysis

1 Introduction

The *Castanea sativa* Mill. species belongs to the *Fagaceae* family and is the only native species of the genus *Castanea* in Europe. Nowadays, its widespread distribution is the result of both natural and anthropogenic factors that affected the species over time (Conedera et al., 2004). Climatic conditions have influenced chestnut distribution since the Pleistocene; during this glacial period, the species was confined to limited and climatically stable areas called refugia (Krebs et al., 2004; Mattioni et al., 2013). Subsequently, during the postglacial period, the natural recolonization of chestnuts started from these areas.

However, chestnut distribution was also influenced by human colonization and migration. In southern Italy, chestnut trees were introduced by the ancient Greeks about 5,000 years ago. Later, the Romans spread this species in Europe (Huntley and Birks, 1983; Bernetti, 1995; Krebs et al., 2004 and 2019; Roces-Diaz et al., 2018).

In the last 20 years, several studies have described in detail the fundamental role of the historical and natural processes that resulted through time in the establishment of the varietal genetic diversity of chestnuts that exists to this day (Martín et al., 2009; Pereira-Lorenzo et al., 2010, 2011; Marinoni et al., 2013; Lusini et al., 2014, Villani et al., 1999; Martín et al., 2012; Fernández-Cruz and Fernández-López, 2016; Mattioni et al., 2013 and 2017). While the genetic pool of the domesticated

47 trees was influenced by human selection, propagation and hybridization (to improve fruit or wood
48 quality traits, as well as resistance to abiotic and biotic stress), wild trees were subjected to natural
49 selection to adapt to different environmental and geographical conditions (Barrett and Schluter,
50 2008; Nishio et al., 2021). In particular, the selective pressure to which wild populations were sub-
51 jected favored different allele pools involved in the trees' adaptation to different regional condi-
52 tions, such as adaptation to drought (Soto et al., 2019; Alcaide et al., 2019; Castellana et al., 2021).
53 Furthermore, wild chestnut genetic variability has also been affected by the proximity of orchards,
54 mainly due to pollen diffusion and male-sterility, which have contributed to the inclusion of local
55 and nonlocal new alleles (Lopez et al., 2021). It is also worth mentioning the importance of non-
56 grafted giant chestnuts as a reservoir of genetic diversity, which represent the basis from which the
57 selection and cultivation process commenced (Pereira-Lorenzo et al., 2019).

58 Despite the different selection process, domesticated chestnuts have preserved most of the diversity
59 found in the oldest wild tree populations (non-grafted giant chestnuts; Pereira-Lorenzo et al., 2019).
60 However, the hybridization conducted by humans improved chestnut genetic variability. For in-
61 stance, the crosses between *C. sativa* and the Asiatic species allowed the creation of new hybrid cul-
62 tivars that are resistant to several pathogens (e.g., to the pathogenic fungus *Gnomoniopsis pascoe*
63 and the Chinese wasp *Dryocosmus kuriphilus*) and tolerant to biotic stress (Sartor et al., 2009 and
64 2015; Dini et al., 2012; Botta et al., 2012; Alcaide et al., 2020 and 2021).

65 Low differences in genetic variability have been observed between chestnut cultivars and wild trees
66 (Pereira-Lorenzo et al., 2019; Bouffartigue et al., 2019 and 2020).

67 Therefore, in this study, we assessed a genetic characterization among chestnut cultivars and wild
68 chestnut trees from the Iberian Peninsula and Italy. The main aims of this research were: a) to inves-
69 tigate the gene pools of chestnut trees from Italy and the Iberian Peninsula based on the reference
70 SSR; b) to estimate the genetic diversity between wild chestnut trees and chestnut varieties.

71

72 ## 2 Materials and methods

73 2.1 Plant material

74 A total of 630 wild chestnut trees and chestnut varieties were analyzed: in particular, 244 were new-
75 ly collected samples and 386 were derived from previous studies (Table S1). More in detail, 520
76 samples were varieties and the remaining 110 derived from wild chestnut trees, as single and isolat-
77 ed trees.
78 13 representative samples of *Castanea pumila, C. crenata, C. mollissima* and the 'Volos' cultivar
79 were added to the analysis in order to collect information on interspecific hybrids versus Italian and
80 Spanish chestnut samples.
81 The 630 genotypes were standardized with the SSR profiles of the unique accessions available in
82 the European Chestnut Database (Pereira-Lorenzo et al., 2017).
83
84 2.2 DNA extraction and PCR amplification of microsatellites (SSRs)
85 Young leaves used for DNA extraction were frozen in liquid nitrogen and stored at -80 ° C or ly-
86 ophilized. The extraction was performed on samples of 0.1-0.5 grams of fresh leaves previously
87 ground in liquid nitrogen, or on 5 mg of ground lyophilized leaves. The DNA was extracted follow-
88 ing the CTAB protocol developed by Maguire et al. (1994). dsDNA was quantified using a
89 Nanodrop^{TM} ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted
90 to 10 ng/µl.
91 The microsatellites chosen were selected from the CsCAT and EMCs series, and OAL (Marinoni et
92 al., 2003; Buck et al., 2003 and Gobbin et al., 2007) and QrZAG (Kampfer et al., 1998) series. The
93 primers were used in multiplex sets according to Pereira-Lorenzo et al. (2017).
94 The PCR reaction was performed in 10 µl final volume containing 6.45 µl of sterile H_2O, 1 µl of
95 GeneAmp® 10X reaction buffer, 0.8 µl of $MgCl_2$, 0.25 of dNTPs and 0.1 units AmpliTaq Gold^{TM}

96 DNA polymerase. Amplification products were sequenced on a 3130 Genetic Analyzer capillary
97 sequencer (Applied Biosystems, USA). The internal GeneScan TM size standard 500 LIZ (-250)
98 was included in each sample. The allele sizes were detected using Peak Scanner TM software (Ap-
99 plied Biosystems). The samples collected were suitably standardized with the alleles found in the
100 European dataset for the 16 SSR being assessed.
101

102 2.3 Cluster analysis

103 The genetic distance between the 630 samples was calculated by using the R software. The con-
104 struction of the genetic distance dendrogram was elaborated using the Unweighted Pair-Group
105 Method (UPGMA) by R software with the function 'hclust, method = 'average'', packages 'adege-
106 nett".
107

108 2.4 Genetic diversity and gene pools structure

109 The genetic diversity indices were assessed by the Cervus software, version 3.0.3 (Kalinowski et
110 al., 2007): the number of alleles per locus (k), the expected and the observed heterozygosity (H_e and
111 H_o), polymorphism information content (PIC) and the probability of the allele null (F-null) were es-
112 timated. A PIC threshold of 0.7 was considered for defining loci as highly polymorphic and in-
113 formative. The frequency of the null alleles (F-null) for each locus was calculated using the maxi-
114 mum likelihood (ML) estimator of Kalinowski (2007) implemented in Cervus. The Marker index
115 (MI) was calculated with iMEC: Online Marker Efficiency Calculator (Amirvousefi et al., 2018).
116 The MI allowed the measurement of polymorphism information for the individual markers used.
117 The STRUCTURE 2.3.3 (Pritchard et al., 2000) software was used to evaluate the genetic pool sub-
118 division of 319 genotypes (varieties and wild trees) and to calculate the estimated membership coef-
119 ficient (Q-value) that indicates the membership of each individual in each cluster. This analysis was
120 conducted with a Bayesian approach combined with the Markov Chain Monte Carlo (MCMC) sim-
121 ulation method and was performed using an "admixture model" and correlated allele frequencies.

122 Following the protocol of Pereira-Lorenzo et al. (2019), 30 replicate runs of STRUCTURE were
123 performed by setting the number of clusters (K) from 1 to 15. Each run consisted of a burning peri-
124 od of 200,000 steps followed by 200,000 MCMC replicates, with the usage options: locprior=0,
125 popinfo = 0, popflag = 0 (Pereira-Lorenzo et al., 2019; Porras-Hurtado et al., 2013).

126 In order to check if the inclusion of hybrid samples affected the STRUCTURE analysis, the hybrid
127 samples were removed, and a second analysis was performed on a total of 306 samples with the
128 same conditions described above (Figure S3). A Q threshold of 0.8 was used to infer an accession to
129 a specific cluster. The ΔK value (defined as the most probable number of clusters in the population)
130 was calculated through Structure Harvester v.09.93 (Earl, 2012) by testing the change of the log-
131 likelihood between K values (ΔK) as described by Evanno (2005). If a sample has a Q-value < 0.8,
132 it is considered an admixed sample.

133

134 ## 2.5 Genetic differentiation

135 To validate the genetic structure revealed by the Bayesian model-based clustering, a multivariate
136 Principal Coordinate Analysis (PCoA) was elaborated with GenAlEx version 6.502 (Peakall and
137 Smouse, 2006). The PCoA representation was determined on the genetic distance measured by Jac-
138 card coefficient, based on the estimates of ΔK from STRUCTURE for 306 samples (hybrid samples
139 were not considered). A set of analysis to estimate the population differentiation was conducted un-
140 der four scenarios: a) the two main groups (Cluster 1 vs Cluster 2) resulting from the Structure
141 analysis; b) the sub-groups (K=3, K=4), c) chestnut varieties versus wild trees (200 vs 106, respec-
142 tively) and d) chestnut varieties and the wild trees separated in the two main clusters (Cluster 1 and
143 Cluster 2). Pairwise F_{ST} values and private alleles (Np) were estimated for the different partitioning
144 levels considered using GeneAlEx version 6.502; missing data were coded as 0. The F_{ST} value
145 ranges between -1 (absent inbreeding, excess of heterozygous) and 1 (non-random reproduction,
146 excess homozygous).

147 The gene flow (Nm) was estimated for the different partitioning levels considered (K=2, 3 and 4)
148 using GenAlEx version 6.502 (Peakall and Smouse, 2012). Hierarchical analysis of molecular vari-
149 ance (AMOVA) was implemented in the GeneAlEx version 6.502 (Peakall and Smouse, 2006) in
150 order to evaluate the genetic variation among and within Clusters. Tests of significance were per-
151 formed using 9999 permutations within the total dataset of 306 samples. The 13 hybrids were re-
152 moved from this analysis.

153 # 3 Results

154 ## 3.1 Genetic variability of the 16 microsatellites

155 The 16 SSRs showed high levels of polymorphism and discriminating power and revealed a total of
156 212 alleles, with an average number of 13.25 alleles per locus (Table 1). The average PIC was
157 0.735, ranging between 0.879 for *CsCAT3* and 0.593 for *EMCs2*. Furthermore, expected
158 heterozygosity varied between 0.889 for *CsCAT3* and 0.619 for *CsCAT1,* with a mean value of
159 0.763 (Table 1). *CsCAT41* was known to amplify two different genomic sites (A and B); for this
160 reason, the *CsCAT41A* locus was removed from the dataset before the analyses (Pereira-Lorenzo et
161 al., 2010). *CsCAT2* and *EMCs38* showed a high frequency value of null alleles (0.209 and 0.118,
162 respectively); consequently, these two loci were removed from the subsequent analysis.

163

164

165

166 **Table 1:** Number of alleles (k), observed (Ho) and expected (He) heterozygosity, polymorphic information content
167 (PIC), null alleles frequencies (F[null]) and marker index (MI) for 319 unique genotypes of *C. sativa* accessions evalu-
168 ated with 16 SSRs.

Locus	k	Ho	He	PIC	F(Null)	MI
CsCAT41B	12	0.690	0.812	0.793	0.071	0.270

CsCAT16	12	0.777	0.812	0.785	0.018	0.262
CsCAT6	19	0.846	0.872	0.858	0.013	0.186
CsCAT1	16	0.596	0.619	0.601	0.022	0.197
CsCAT3	26	0.803	0.889	0.879	0.050	0.165
QrZAG96	12	0.596	0.718	0.694	0.082	0.311
EMCs15	7	0.599	0.661	0.605	0.053	0.385
EMCs38	19	0.693	0.883	0.871	0.118	0.204
EMCs2	6	0.624	0.661	0.593	0.030	0.427
EMCs22	10	0.652	0.682	0.654	0.020	0.301
CsCAT2	16	0.555	0.855	0.841	0.209	0.240
CsCAT17	13	0.771	0.844	0.827	0.042	0.284
CsCAT14	10	0.721	0.753	0.711	0.015	0.313
CsCAT15	11	0.665	0.666	0.605	-0.003	0.266
CsCAT8	11	0.727	0.843	0.821	0.068	0.341
OAL	12	0.586	0.651	0.629	0.048	0.268
Mean	13.25	0.681	0.763	0.735	0.005	0.257

169

170 3.2 Cluster analysis

171 A dendrogram was constructed using the UPGMA method with R software to evaluate the genetic
172 diversity and relatedness between the 630 wild and grafted chestnut trees.

173 Clustering according to the UPGMA method allowed the authentication of accessions (true-to-type
174 cultivars), as well as the indication of possible homonyms, synonyms and incorrect denominations.

175 The 630 samples corresponded to 319 unique chestnut genotypes (Figure S1).

176

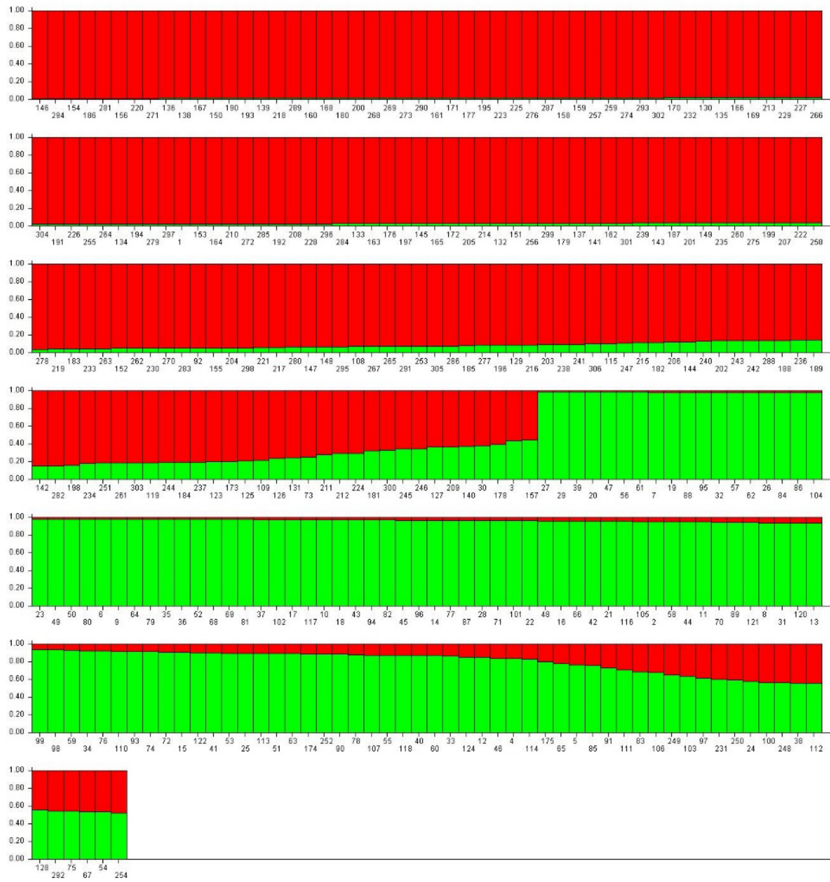
177 3.3 Gene pools

178 The genetic structure of the 319 unique genotypes (including varieties and wild chestnut trees) was
179 evaluated using the 14 loci that did not show the presence of null alleles. In the first set of STRUC-
180 TURE analysis (Figure S2), the ΔK statistics gave a maximum value of K=2 (ΔK = 78.61), alt-
181 hough a small peak of ΔK was also observed for K=3 (ΔK=18.42), K=6 (ΔK=6.21) and for K=8
182 (ΔK=6.05).

183 For K=2, genotypes were grouped into two main clusters with a clear distinction between Spanish
184 genotypes, represented by Cluster 1 (with 102 accessions), and Italian genotypes, represented by
185 Cluster 2 (with 163 accessions). The threshold for membership determination was Q > 80%. Addi-
186 tionally, 54 admixed samples were found (Q < 80%) (Figure 1A).

187 For K=3, a further separation of hybrid samples was observed. Cluster 3 included the Euro-Chinese
188 hybrids (Figure 1B) and some significant Italian cultivars (‘Marrone Fiorentino’; ‘Madonna’, ‘Lu-
189 cente’ and ‘Inserta’) as well as two varieties from the Andalucía region (‘Tomasa’ and ‘Capilla’).

190



191 **Figure 1A:** Representation of 319 samples for K=2 by STRUCTURE Software. Each individual is represented by a

192 vertical line and each cluster by different colors: Cluster 1 in red, Cluster 2 in green.

193

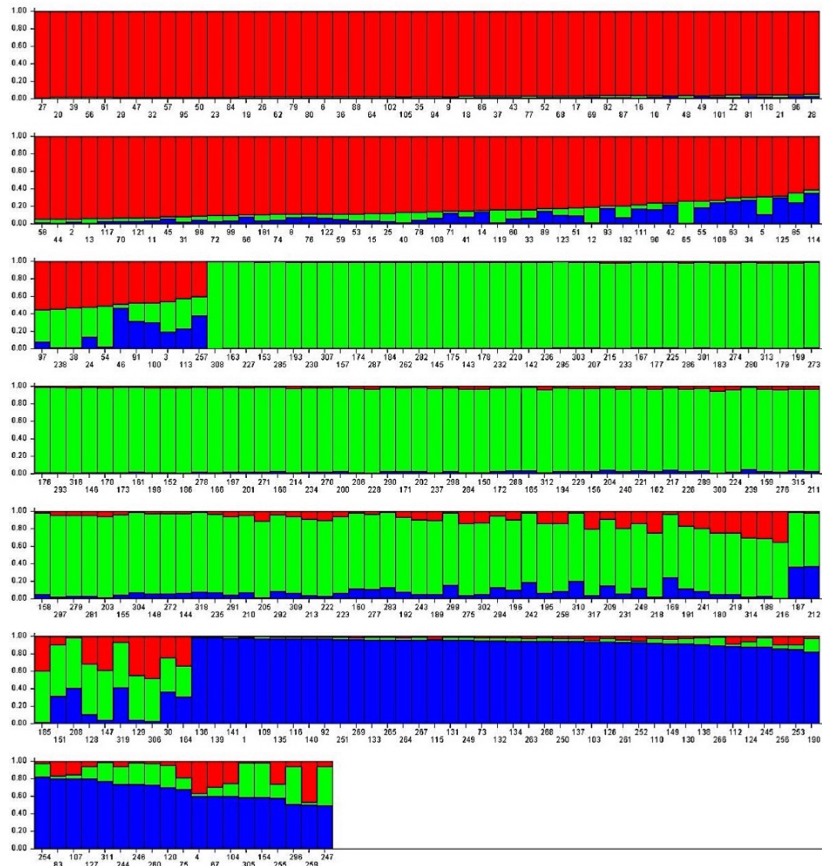


Figure 1B: Representation of 319 samples for K=3 by STRUCTURE Software. Each individual is represented by a vertical line and each cluster by different colors: Cluster 1 in red, Cluster 2 in green and Cluster 3 in blue.

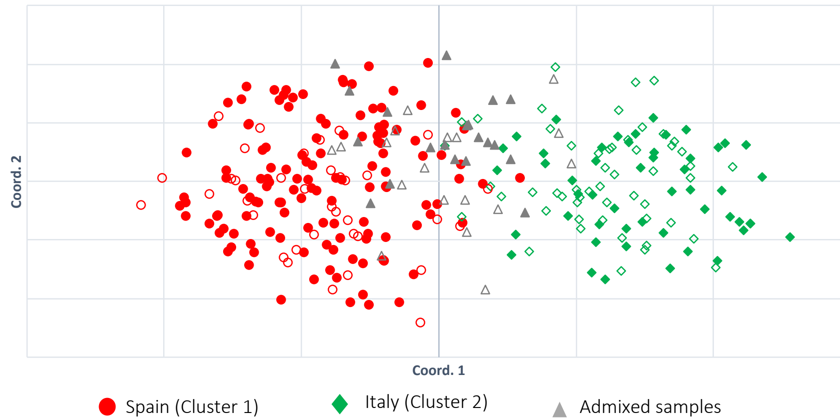
3.4 Genetic structure of chestnut accessions of the Iberian and Italian Peninsulas (varieties and wild trees)

The results confirmed the clear separation between the two main clusters (Cluster 1 represented by 167 Spanish samples; Cluster 2 represented by 104 Italian and 35 admixed samples). The comparison of chestnut varieties and wild chestnut trees within each cluster did not highlight genetic structural differences (Table S3). The separation for K=3 and K=4, with ΔK=27.34 and 25.72 respectively, was also considered (Figure S3, Table S3). Accordingly, the separation between the Italian and Spanish varieties was also maintained in further subdivisions. The Italian cluster was represented as follows: Cluster 1 for subdivision K=3 included 89 samples; Cluster 4 for subdivision K=4 had 86 samples. The Italian cluster included both wild chestnut trees (such as samples named ‘Matildico’,

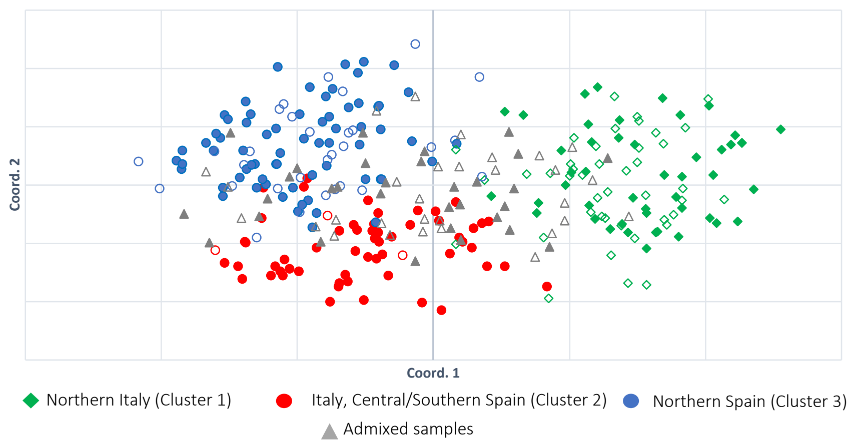
209 ‘Legno’) and varieties from the Tuscan-Emilian Apennines (such as samples named ‘Pastanese’,
210 ‘Ceppa’, ‘Pistolese’, ‘Piusela’ and ‘Lisanese’).
211 An important group of chestnut varieties was Cluster 2 for subdivision K=3 with different relevant
212 varieties: (e.g. ‘Marrone Fiorentino’) from northern Italy (Emilia-Romagna, Trentino Alto Adige
213 and Piedmont); (e.g., ‘Riggiola’) from southern Italy (Calabria and Campania); (e.g., “Temprana”)
214 from southern Spain (Canary Islands, Extremadura, Andalucía). Varieties from Galicia Asturias and
215 Andalucía, such as “Luguesa”, “Longal”, “Temprana” and “Miguelina”, as well as “Martahiña”,
216 were also represented in Cluster 2 (Table S3).
217 Most of the varieties contained in Cluster 2 for subdivision K= 3 were also included in Cluster 1 for
218 subdivision K=4. In Cluster 2, only a few wild chestnut trees were present (Table S3).
219 Finally, Cluster 3 for subdivision K=3 included the main varieties from northern Spain: Galicia
220 (‘Famosa’, ‘Inxerta’), Castilla-León (‘Negral’), Asturias (‘Parede’, ‘Rapuca’ and ‘Chamberga’),
221 Cantabria, some accessions from Extremadura (‘Verata’) and from Canary Islands (‘Mollar’, ‘Mu-
222 lata’ and ‘Armentina’; Table S3). In addition, most part of the wild chestnut trees from Galicia,
223 Castilla-León and Cantabria were grouped in Cluster 3 (such as ‘Pesaguero’, ‘Alcobilla’,
224 ‘Peixeroos’).
225
226 3.5 Genetic differentiation
227 The PCoA analysis performed on subdivision K=2 corroborated the results of the STRUCTURE
228 analysis by showing a clear separation between Italian (green) and Spanish (red) accessions (Figure
229 2A). Some admixed samples were observed as reported by the STRUCTURE analysis.
230 Results obtained for subdivision K=3 separated northern (blue) from southern (red) Spanish varie-
231 ties. The northern Italian cluster appeared particularly well separated (green) from the two Spanish
232 clusters (Figure 2B).
233 Furthermore, a larger number of wild chestnut trees was found in the northern Spanish and Italian
234 clusters than in the one from southern Spain, in which most samples were varieties (Figure 2B).

235 Subdivision K=4 showed the presence of two further (sub) clusters (mainly chestnut varieties) in
236 southern Spain: the first is a cluster deriving from southern Spain and Italy and the second a cluster
237 deriving from central-southern Spain (represented by Cluster 1 and Cluster 2) (Figure 2C).
238 The genetic differentiation between the two main clusters was F_{ST} = 0.077, $P < 0.001$ (Table S4.A),
239 suggesting a genetic structure for the chestnut at Italian and Spanish level, also confirmed by the
240 AMOVA results (8%) (Table 2A). A higher gene flow (Nm) (9.235) was detected for subdivision
241 K=2. In addition, the number of private alleles detected for subdivision K=2 and the observed val-
242 ues are quite similar (0.8571 and 0.7142 respectively; Table S5).
243 Similar AMOVA results were found for K=3 and K=4, with a 6% and 7% of variance component
244 among the populations, respectively (Table 2, B and C). In contrast, high gene flows (Nm) (6.118
245 and 3.302 respectively) were detected for subdivisions K=3 and K=4.
246 The largest differentiation between pairs of groups was found between the northern Italian cluster
247 (Cluster 4), with samples mainly from the Tuscan-Emilian Apennines, and the northern-central
248 Spanish cluster (Cluster 3) for K=4 (F_{ST}=0.133, $P<0.001$), as shown in Table S4.C.
249 A high F_{ST} value was observed also between Cluster 1, containing cultivars from Italy and southern
250 Spain, and Cluster 4, represented by the Italian cluster with F_{ST}=0.113, $P<0.001$; similarly, between
251 Cluster 1 and Cluster 2, which included central Spain's varieties (F_{ST}=0.112, $P<0.001$).
252 In addition, AMOVA analysis, conducted between wild trees and chestnut varieties, showed no dif-
253 ferences between them, in agreement with the STRUCTURE division into two main clusters (Table
254 2. D and E). The variance components among populations were 1% and 5% respectively, confirmed
255 also by the F_{ST} index (0.0012 with $P<0.001$, Table S4. D and E). The Nm values between wild
256 chestnut trees and varieties and between the two main clusters subject of the STRUCTURE analysis
257 were 8.112 and 6.364 respectively. Notably, the number of private alleles (Np) in chestnut varieties
258 (0.9285) was higher than the number of private alleles in wild chestnut trees (0.5714, Table S5).

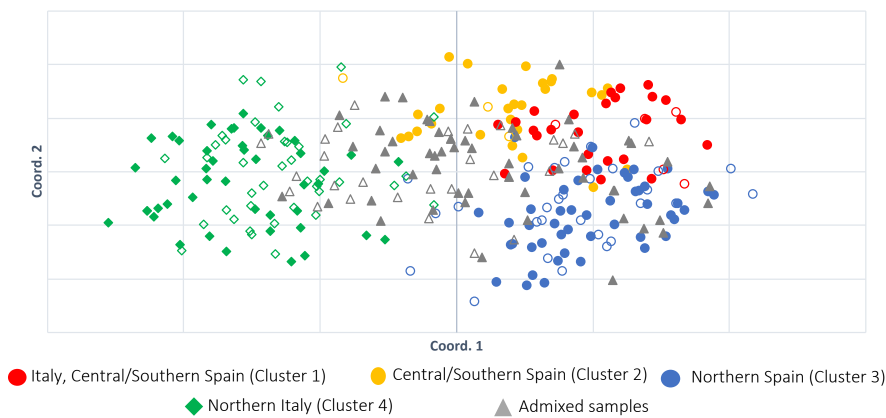
A) Principal Coordinates (PCoA) K=2



B) Principal Coordinates (PCoA) K=3



C) Principal Coordinates (PCoA) K=4



259

260 **Figure 2:** Principal Coordinate Analysis (PCoA) based on polymorphism at 14 SSR loci for 306 unique genotypes. A),
261 K=2; B), K=3; C) K=4. Accession color reflects the consistent assignment using Bayesian analysis to the sub-groups
262 defined in Fig. 3. Wild chestnut trees are represented by empty symbols inside the sub-groups.

263

264 **Table 2:** Analysis of molecular variance (AMOVA) based on the 14 SSR loci of 306 chestnut accessions defined by

265 STRUCTURE analysis. All estimates were highly significant - P<0.001.

	Populations	**Df**		**Variance components (%)**		**p-value**
		Among Populations	Within Populations	Among Populations	Within Populations	
A)	Structure Cluster K=2, No hybrids included (306 samples)	2	609	8	92	0.077
B)	Structure Cluster K=3, No hybrids included (306 samples)	3	608	6	94	0.060
C)	Structure Cluster K=4, No hybrids included (306 samples)	4	607	7	93	0.070
D)	306 chestnut trees divided in chestnut varieties and wild	1	610	1	99	0.012
E)	306 chestnut trees divided in chestnut varieties and wild in Cluster 1/Cluster 2	4	607	5	95	0.048

266

267
4 Discussion

268 Our results confirmed the high degree of variability of *C. sativa* and the selected 16 SSRs markers
269 as powerful tools to evaluate the genetic diversity of EU chestnut germplasm (Pereira-Lorenzo et
270 al., 2011, 2017; Martín et al., 2017a). In particular, the *CsCAT3* (PIC=0.879) and *EMCs15*
271 (PIC=0.604) loci appeared to be the most and least informative loci respectively, as previously re-
272 ported by Pereira-Lorenzo et al. (2010, 2011) and Martín et al. (2012). The average number of al-
273 leles per locus in this study was 13.25 for 16 SSRs, as in Pereira-Lorenzo et al. (2017), with 8.92
274 average using 24 SSRs.

275 Our results highlighted the complex structure and genetic diversity of chestnut trees. Genetic diver-
276 sity is linked to climatic conditions, mainly temperature and precipitation gradient (Pereira-Lorenzo
277 et al., 2010) and to the domestication process carried out by humans through the centuries (Pereira-
278 Lorenzo et al., 2011, 2019).

279 Hybridization could also have played an important role in the diversification process, as previously
280 suggested by Pereira-Lorenzo et al. (2011), and, furthermore, it explains the great diversity found in
281 small geographical areas such as the Tuscan-Emilian Apennines (central-northern Italy) and Galicia
282 (northern Spain), as shown by the STRUCTURE and PCoA analysis. In these regions, wild and
283 domesticated chestnuts were found to be genetically similar and cannot be separated. This is in

284 agreement with previous studies, in which no substantial differences between chestnut varieties and
285 wild chestnut trees were found (Fst=0.007 in Pereira-Lorenzo et al., 2019; Fst=0.008 in Bouffar-
286 tigue et al., 2020). This evidence is supported by the AMOVA analysis that showed a low F_{ST} value
287 among wild trees and cultivars (F_{ST}= 0.012 with P<0.001). The Fst and the Nm values observed in
288 samples from the Tuscan-Emilian Apennines and Galicia can be explained considering these areas
289 as the probable sites where the domestication process started, favored by the high number of au-
290 tochthonous chestnut populations. In addition, private alleles were also detected in higher values in
291 chestnut varieties, due probably to selection practices, and they have evolutionary significance (Petit
292 et al., 1998).
293 Our results therefore highlight a low gene flow between chestnut varieties and wild trees. The gene
294 flow between wild trees and chestnut varieties is maintained by changes in forest use over time and
295 the practices related to how the forests were used (Pereira-Lorenzo et al., 2019).
296 At least three major results were obtained by the STRUCTURE analysis.
297 Firstly, the STRUCTURE analysis evidenced a clear separation between the Spanish and the Italian
298 chestnut trees with admixed samples. Similar results, differentiating Spanish and Italian varieties,
299 were previously reported for adaptive markers (Martín et al., 2017b).
300 The genetic differentiation between the two clusters and the admixed samples was low (F_{ST}=0.019
301 and 0.032, P<0.001 respectively). In addition, the AMOVA analysis which compared varieties and
302 wild trees within the two clusters did not show genetic structural differences (F_{ST} =0.012, P<0.001).
303 The large number of admixed samples can indicate a hybridization between the two clusters as
304 suggested also by Pereira-Lorenzo et al. (2012). Furthermore, the aforesaid author evidenced that
305 the hybridization process occurred before the 15th century by considering the oldest giant trees from
306 Andalucía with Italian genetic background (Pereira-Lorenzo et al., 2019).
307 Secondly, the STRUCTURE analysis evidenced a separation among samples belonging to the
308 northern and central/southern Spanish Clusters. In particular, the Northern Cluster contained most
309 of the wild chestnut trees while the Central/Southern Cluster included far more chestnut varieties,

310 probably due to human selection (and vegetative propagation of the selected genotypes) related to
311 the use of new seedlings from local cultivars selected for the superior traits of their nuts (Auge and
312 Brandl, 1997; Forneck, 2005; Pereira-Lorenzo et al., 2010). In this respect, Pereira-Lorenzo et al.
313 (2010), reported that the distribution of seedlings from the main cultivar groups from the northern
314 and central Iberian Peninsula was used to create new orchards in southern Spain, in Andalucía and
315 the Canary Islands (e.g., the "Longal", "Reborda" and "Dieguina" cultivars). Our results confirmed
316 the relationship between "Longal" and the main different varieties from southern Spain, such as
317 "Laga", "Temprana" and "Pelona", and from Extremadura, with "Injerta". This was also in line
318 with previous studies (Pereira-Lorenzo et al., 2006, and Costa et al., 2008), which identified "Lon-
319 gal" as a cultivar used for genetic contribution to create new cultivars in different regions of Spain.
320 This may also explain the huge number of admixed samples between the sub-clusters being as-
321 sessed.
322 Finally, the STRUCTURE analysis evidenced that part of the varieties from southern Spain (Anda-
323 lucía) shared a higher number of alleles with both varieties from southern Italy (Calabria and Cam-
324 pania regions) and with "Marrone Fiorentino", the most important northern Italian variety.
325 As shown in Figure 3, northern Italy produced a high introgression (30% of the genotypes) in cen-
326 tral-southern Spain and an even higher introgression in central-southern Italy (50%). Moreover, in-
327 trogressions from both the north and the south of Italy were also noticed in northern Spain (16%),
328 with 5% from northern and 11% from southern Italy.
329 The above results are in line with Pereira-Lorenzo et al. (2019), who supports an early introduction
330 of chestnut cultivations from Italy into Spain. In particular, the introduction of chestnut cultivations
331 started in the Andalucía and Extremadura regions, with contacts also in Castilla-León and Galicia
332 (see the results related to the "Luguesa" cultivar, which was included in Cluster 2 for K=3 and
333 Cluster 1 for K=4 with the main southern Italian varieties).
334
335

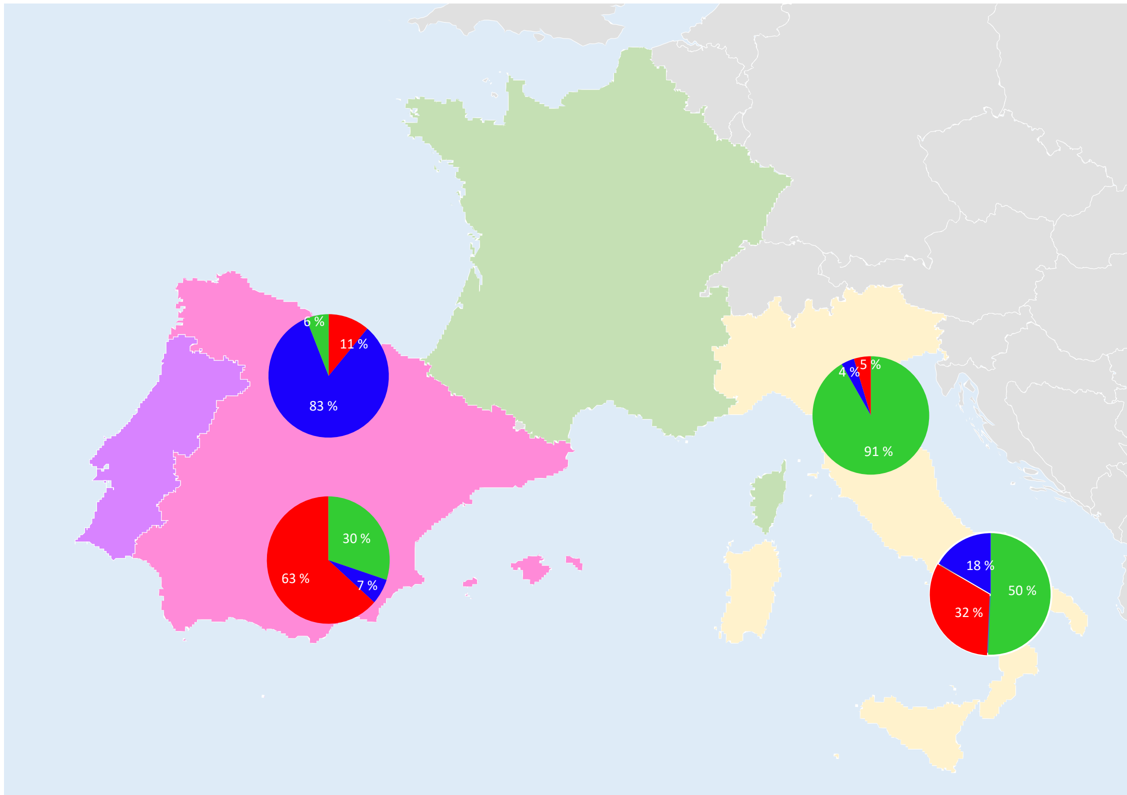


Figure 3: Gene pool distribution of chestnut cultivars for K=3 between the north and the south, both of Italy and Spain. Green – Mainly northern Italian and France (Cluster 1); Red – Italy, central and southern Spain (Cluster 2); Blue – northern Spain (Cluster 3).

5 Conclusions

This study contributes to improving knowledge on the genetic relationships between chestnut varieties and wild chestnut trees in the Iberian and Italian Peninsula and to expanding the Chestnut European Genetic Dataset. The genetic variations between and within Italian and Spanish clusters, including both chestnut varieties and wild trees, reflect a combination of historical migrations and selection processes. This is highlighted by the high number of admixed in relation to the chestnut populations of central-southern Spain and central-southern Italy. Adaptation to different environments and hybridization led to a wide genetic variability in limited areas such as the Tuscan-Emilian Apennines and the Galicia region. In these areas, no genetic structure differences between wild chestnut trees and chestnut varieties was found.

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577

578 ## Supporting information

579 **Table S1:** unique genotypes analyzed in this study (319 samples).

580

581 **Table S2:** list of the different varieties collected with their countries of origin, the STRUCTURE
582 subdivision for K=2 and K=3 for 319 samples. A Q threshold of 0.8 was used to infer an accession
583 to a specific cluster.

584

585 **Table S3:** list of the different varieties collected with their countries of origin, the STRUCTURE
586 subdivision for K=2, K=3 and K=4 for 306 samples (no hybrids included) and varieties/wild subdi-
587 vision. A Q threshold of 0.8 was used to infer an accession to a specific cluster.

588

589 **Table S4**: pairwise estimate of Fst value based on the 14 SSR loci of 306 chestnut accessions de-
590 fined by STRUCTURE analysis: A), K=2; B), K=3; C) K=4; D) the total population (n= 306) di-
591 vided into wild trees and chestnut varieties; E) wild trees between chestnut varieties in K=2. All es-
592 timates were highly significant - P<0.001.

593

594 **Figure S1:** UPGMA tree indicating the relationships among 630 chestnut samples.

595

596 **Figure S2:** Estimates of ∆ k calculated based on Evanno et al., (2005), based on k-subdivision for
597 319 samples.

598

599 **Figure S3:** Estimates of ∆ k calculated as described by Evanno et al., (2005), based on k-
600 subdivision for 306 samples.