Genetic Characterization of Tuscan Chestnut Germplasm: Genetic and Genotypic Variation among Populations of Three Different Areas

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Abstract

The study was conducted to characterize 131 chestnut plants collected in three different areals of the Tuscany region (Garfagnana, Colline Metallifere, Amiata). The three areals are well separated without continuous chestnut presence. Molecular analysis was performed with 8 microsatellite markers. The 131 plants belonged to 39 names, only 1 of which was present in all the tree population while 4 were present in two population. The results of the fingerprinting showed that identical genotypes were present only within each population. The final number of genotypes was set to 102, once eliminated homonyms and synonyms. The three presented populations differed at the genetic level, although the plants of Colline Metallifere and Amiata shared some alleles. At the genotypic level the three populations differed statistically. Hence, the populations presented different genetic pools and may represent independent sources of variation in breeding. For one of the loci a high level of homozygosity was found in all the populations suggesting a possible correlation between this marker and a gene selective against heterozygosis.

INTRODUCTION

Sweet chestnut (*Castanea sativa* Mill.) is considered to be native of Europe, but it has undergone a rapid expansion to the present areas of cultivation mainly during the Roman period (Zohary and Hopf, 1988). The genetic variability of chestnut has been assessed both in natural populations and in cultivated varieties (Fineschi et al., 1994; Villani et al., 1994, 1999). These studies have shown that genetic diversity varies consistently due to natural and artificial selection and local effects of isolation by distance (Casoli et al., 2001).

The improving of a sustainable development of chestnut cultivation through the exploitation of local germplasm has become of great importance and the Tuscany region has implemented a legislation for the protection of genetic autochthonous resources (Turchi, 2005). Although, little is known about the true genetic variability within the Tuscan chestnut germplasm and so far only morphological traits have been used for characterization.

In the present work eight microsatellite markers were chosen to characterize the chestnut germplasm of Tuscany belonging to three different and separated areals of Tuscany; namely Garfagnana, located in the north part of the region, Colline Metallifere, in the center; Amiata, in the southern part (Fig. 1). The aim of the work was to detect errors in accession names in order to discover cases of homonymy and synonymy as well as to asses genetic diversity among and within the three geographically separated populations.

MATERIALS AND METHODS

Chestnut trees were located in the three chestnut-growing areas using local germplasm collections or long-time selected mother plants. In July 2008, young leaves were collected and total genomic DNA extracted from 100 mg of fresh tissue using a

modified Doyle and Doyle (1990) method. All the polymerase chain reactions (PCRs) with the DNA extracted from the accessions were performed on a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) in 25 µl volume containing 5 µl of DNA (10 ηg/µl) and 20 µl mix. The eight couples of primers CsCAT1, CsCAT3, CsCAT6, CsCAT16, CsCAT17, BH1, BH2 and BH4 were labeled with 6FAM™, HEX™, NED fluorescent dyes for multiplexed genotyping. Cycling conditions were the same for all loci. Initially, DNA was denatured for 3 min at 94°C followed by 35 cycles of 94°C for 30 s, Ta in relation to the used primer pairs for 30 s, and 72°C for 30 s. A final 8-min extension at 72°C was included. Allele sizing was performed by Fragment Profiler program after sequencing on MegaBACE™ 500 capillary sequencer (GE Healthcare).

The software NTSYSpc (Exeter Software, Setauket, NY) was used to calculate the genetic distance among accessions with the production of a similarity parameter based on the number of shared bands (Lynch, 1990). Sequential agglomerative hierarchical nested cluster analysis with unweighted pair group method of clustering (UPGMA) was successively applied and the tree plot procedure of the same package was finally used to provide a graphic representation of Lynch's similarity index data, from which relationships among accessions may be deduced. Number of alleles per locus, allele frequencies, expected (H_E) and observed (H_O) heterozygosity (Nei, 1978) Hardy-Weinberger (HW) exact test, genetic (Fst) and genotypic differentiation (Fis) were calculated using the GENEPOP software (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Twenty two accessions had a fingerprinting identical to others as a probable result of clonal propagation, but plants with identical fingerprinting were present only within each populations. Among these accessions we also found cases of homonymy and synonymy. After this preliminary work the number of final genotypes representing the variability inside the germplasm of the three different areas was set to 102.

Total expected heterozygosis (Table 1) was high varying from 0.653 for locus CsCAT1 to 0.893 for locus BH4. Excess of heterozygosis was significant for the locus CsCAT6 (p=0.032) and CsCAT17 (p=0.000), while the locus BH2 showed a significant (p=0.007) heterozygosis deficiency. Garfagnana's population showed a lower genetic diversity and allelic richness than the other two (Table 2). Mean differentiation value F_{ST} (Weir and Cockerman, 1984) ranged from 0.053 to 0.1493 (Table 3). The population of Garfagnana showed a high value of differentiation with the other two, especially with that one of Amiata which is the most geographically distant. Pairwise lower F_{ST} value was found between Amiata and Colline Metallifere populations. At the genotypic level the three populations differed statistically, since the analysis performed by exact G test (log-likelihood ratio) for each population pair across all loci (Fisher's method) gave a p-value uqual to zero (data not shown). The population of Garfagnana again differed from the other two at all loci (Table 4) while Colline Metallifere and Amiata were not differentiated at locus CsCAT 3 and BH4.

CONCLUSIONS

The total genetic diversity found among the sweet chestnut population was in the range of other long-lived woody perennials (Bruschi et al., 2003). Although only populations from a restricted geographical area were investigated significant differences in R_{ST} were observed. In particular, the differentiation value of Garfagnana suggests that isolation by distance could have played a role in the genetic structure of the studied populations. The more closely adjacent populations (Colline Metallifere and Amiata) shared some alleles and some genotypes at two loci, indicating a probable interpopulation pollen flow. Average gene heterozygosity (He) did not differ among the three populations at the microsatellite level. Examining the loci separately, a significant excess of homozygosis (p level varying from 0,007 to 0) was found at locus BH2 in all the populations, suggesting a possible connection with this marker to a gene selective against the heterozygosis. The differences in the genetic pool among the three populations can be

positively used in breeding as they may represent independent sources of variation. From a conservation perspective the research enabled to recognize the presence of duplication, homonyms and synonyms, reducing the number of accession to be further characterized and introduced in the chestnut germplasm collections of Tuscany.

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Tables

Table 1. Characterization of the eight microsatellite markers used for the fingerprinting of Tuscany chestnut germplasm. Value of observed (Ho) and expected (He) heterozygosis and mean number of alleles (MNA) averaged over the three populations. Total He and NA were calculated by grouping all the accessions. The values in parenthesis are standard deviations.

Locus	Ho	He	MNA	He total	NA total
CsCAT 1	0.6665 (0.100)	0.6439 (0.056)	6.7 (0.9)	0.653	8
CsCAT 3	0.6641 (0.072)	0.7476 (0.056)	8.7 (0.8)	0.757	12
CsCAT 6	0.8876 (0.045)	0.8084 (0.056)	8.7 (1.2)	0.869	13
CsCAT 16	0.6759 (0.154)	0.6667 (0.056)	6.0 (0.8)	0.697	8
CsCAT 17	0.8806 (0.082)	0.7425 (0.056)	7.7 (1.7)	0.826	14
BH1	0.7219 (0.077)	0.7630 (0.056)	7.3 (1.7)	0.813	10
BH2	0.3039 (0.203)	0.5679 (0.056)	5.7 (1.2)	0.771	9
BH4	0.8556 (0.065)	0.8422 (0.056)	14.7 (2.5)	0.893	20

Table 2. Genetic variability within the three populations of Tuscan chestnut. Number of accessions (n), observed (Ho) and expected (He) heterozygosis and mean number of alleles (MNA) averaged over all the eight microsatellite loci. The values in parenthesis are standard deviations.

	n	Ho	He	NA
Amiata	27	0.7488 (0.177)	0.7451 (0.056)	8.5 (2.4)
Colline Metallifere	41	0.6883 (0.177)	0.7395 (0.103)	9.3 (3.7)
Garfagnana	34	0.6838 (0.257)	0.6824 (0.135)	6.9 (2.4)

Table 3. Genetic differentiation between Tuscan chestnut populations expressed by mean pairwise F_{ST} value.

Population	Amiata	Colline Metallifere
Colline Metallifere	0.0530	
Garfagnana	0.1493	0.1155

Table 4. *p*-values of genotypic differentiation performed for each population pair by exact G test.

Locus	Population		
	Colline Metallifere Amiata	Garfagnana Amiata	Colline Metallifere Garfagnana
CsCAT 1	0,01709	0,00011	0,03041
CsCAT 3	NS[z]	0,04595	0,00066
CsCAT 6	0,00000	0,00000	0,00000
CsCAT 16	0,02009	0,00000	0,00000
CsCAT 17	0,00996	0,00000	0,00000
BH1	0,00016	0,00000	0,00031
BH2	0,00518	0,00000	0,00000
BH4	NS	0,00073	0,00000

[z] NS, not significant (P>0.05)

Figures

Fig. 1. Map of Tuscany. Genetic characterization was performed on chestnut populations located at the three circled zones.

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