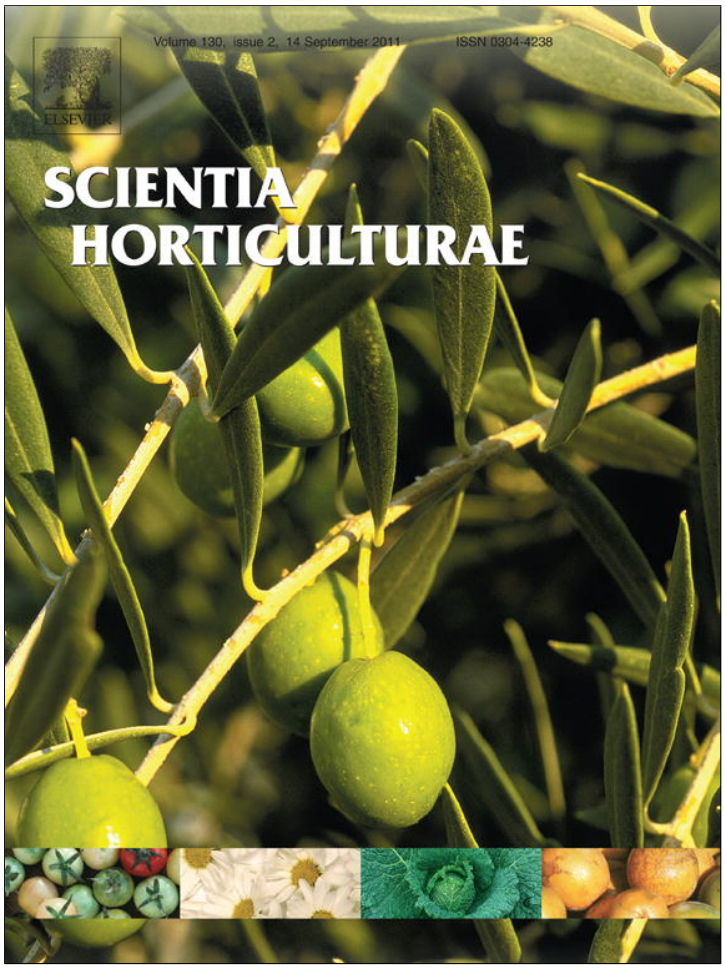

Scientia Horticulturae 130 (2011) 459–467

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Molecular characterization of chestnut plants selected for putative resistance to *Phytophthora cinnamomi* using SSR markers

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ARTICLE INFO

Article history:
Received 28 May 2011
Received in revised form 18 July 2011
Accepted 20 July 2011

Keywords:
Castanea sativa
Microsatellites
Ink disease resistance
Interspecific hybrids
Identification

ABSTRACT

A set of nine short sequence repeat (SSR) loci was used for the molecular characterization of 32 accessions of 15 chestnut trees selected in the field because of their putative resistance to the ink disease caused by *Phytophthora* spp. The goal of the present study was to determine the genetic identity of those selected European chestnut trees (*Castanea sativa*) or interspecific hybrids, considering that hybridization programs between European chestnut and Asiatic species (mainly Japanese chestnut, *Castanea crenata*) have been carried out in Galicia (Spain) since the early 20th century. The results showed that the analyzed SSR loci were useful to discriminate three Asiatic and the European species of *Castanea*. The joint information provided by a factorial correspondence analysis (FCA) and the presence of privative alleles allowed the putative molecular assignment of the selected plants to a certain identity. Most of them were determined as hybrids between *C. crenata* and *C. sativa*. The individuals coded C036 and C048 were assigned, with a high probability, to *C. sativa* due to their clustering with accessions of this species and because they had a number of privative alleles of this species. Only a few individuals could not be assigned to any particular genotype.

1. Introduction

The genus *Castanea* includes 13 species distributed throughout the temperate region of the Northern Hemisphere, and it comprises five Asiatic, seven American and one European species. The European chestnut (*Castanea sativa* Mill.) is considered to be the only species native to Europe, and it is one of the multipurpose tree species of major economic importance in the Mediterranean region (Casasoli et al., 2001).

The natural distribution area of the European chestnut extends from the Caucasus through Turkey, Greece, and the Balkans to Italy, France, Spain, Portugal and South England (Marinoni et al., 2003). In Spain, it can be found in Catalonia, Andalusia and the central and northern regions.

This species was threatened due to the spread of the ink disease during the 19th and 20th centuries. Ink disease is caused by the fungi *Phytophthora cinnamomi* and *Phytophthora cambivora*, which are introduced pathogens associated with the mortality of European chestnut trees (Miranda-Fontaíña et al., 2007). In Spain, the consequences of the ink disease have been so devastating that, since 1875, up to 70–80% of the original natural chestnut populations have disappeared (Vieitez, 1981). The disease spread especially rapidly in the coastal Atlantic region of Galicia (north-western Spain) because *P. cinnamomi* attacks chestnut trees growing on the acid soils and the humid climate of this region (Miranda-Fontaíña et al., 2007).

In Galicia, a program aimed at obtaining hybrids resistant to the ink disease was initiated by Gallastegui (1926) and continued by Urquijo until the middle of the 20th century (Urquijo, 1944). In these studies, *C. sativa* was hybridised with a Japanese chestnut (*Castanea crenata*) and, to a lesser extent, with a Chinese chestnut (*Castanea mollissima*) because of the high level of ink disease resistance described in these species. However, the obtained hybrids were not well adapted to the Atlantic climatic conditions because they lacked the adaptation to summer droughts and winter colds (Pereira-Lorenzo et al., 2010). Thus, the use of hybrid clones is appropriate for plantations in the coastal region of Galicia (a Provenance region named RP1) but not in the Galician Inner Plateau and Mountains (Provenance region named RP2), where the main areas of chestnut cultivation in Galicia (Trives, Viana, Valdeorras, Verín, Allariz-Maceda and Celanova) are located. Therefore, new strategies are being adopted, such as selection of resistant trees in affected populations or the use of resistant hybrids of *C. sativa* × *C. crenata* as rootstocks (Fernández-López and Alía, 2003).

Abbreviations: FCA, factorial correspondence analysis; SSR, short sequence repeat; AMOVA, analysis of molecular variance.

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doi:10.1016/j.scienta.2011.07.020

Likewise, between the years 2000 and 2005, the Agroforest Breeding Department at TRAGSA Company conducted a field selection of adult genotypes of *C. sativa* based on their apparent resistance to *Phytophthora* spp. The screening was performed over an area of 18,808.84 ha included in the Bohuier's regression areas 1 and 2, in Galicia (Bouhier, 1979). These areas had a strong presence of ink disease, and chestnuts were the first or second most abundant species. As a result of this effort, several clones with various levels of tolerance have been selected.

To be commercialized, this selected material must be identified and included in the Spanish National Catalogue of Forest Base Materials. Therefore, the selected plants were micropropagated and tested for resistance against *P. cinnamomi*. To properly identify the selected plants as *Castanea* spp. or as interspecific hybrids a molecular characterization protocol should be developed.

Traditional methods of chestnut cultivar identification are based on geographical origin (Fineschi, 1988) or morphology, ripening period and type of use (Gobbin et al., 2007). However, chestnut species display close morphological traits (Bourgeois et al., 2004), making difficult distinguishing hybrids from pure individuals. In addition, these observations could be error-prone due to environmental variations affecting the expression of genetic traits (Marinoni et al., 2003). Moreover, information on genetic identity is scarce and dubious, making cataloguing difficult and unreliable (Martin et al., 2009). The development of DNA markers has taken advantage of the available polymorphism, enabling the construction of genetic maps and providing tools for cultivar identification (Marinoni et al., 2003).

Recently, SSRs (simple sequence repeats, or microsatellites) have shown their potential for DNA fingerprinting because of their abundance, high degree of polymorphism, large number of genotypes (alleles) per locus and co-dominant inheritance. The use of multi-allelic, co-dominant markers for the analysis of heterozygous species is extremely useful because it allows individuals to be uniquely genotyped (Powell et al., 1996).

Several SSR markers have been isolated and characterized in species belonging to the *Fagaceae* family (Barreneche et al., 1998; Kampfer et al., 1998; Steinkellner et al., 1997; Tanaka et al., 1999), including different chestnut species (Buck et al., 2003; Gobbin et al., 2007; Marinoni et al., 2003; Yamamoto et al., 2003). The development of these SSR markers and the cross-transferability to phylogenetically related species (Barreneche et al., 2004) have allowed to study the diversity and genetic relationships in the genus *Castanea* (Aldrich et al., 2003; Botta et al., 2006; Gobbin et al., 2007; Pereira-Lorenzo et al., 2010; Tanaka et al., 2005; Yamamoto et al., 2003). Furthermore, the first genetic linkage map of the European chestnut based on molecular and isozyme markers has been already published (Casasoli et al., 2001)

The objective of our study was to test a set of SSR markers for the molecular characterization of chestnut trees selected for their putative resistance to the ink disease. The results would allow establishing a reliable and reproducible molecular method for determining the genetic identity of selected plants belonging to *C. sativa* or interspecific hybrids well adapted to the climatic conditions of one of the most important regions of chestnut cultivation. Finally, the identification of selected plants could facilitate their certification and inclusion in the Spanish National Catalogue of Forest Base Materials for commercialization purposes.

2. Materials and methods

2.1. Plant material

In this study a total of 125 *Castanea* accessions were tested. To determine the SSR profile of four *Castanea* species, a first set of 85 accessions from these were tested, distributed as follows:

- Thirty-three accessions belonging to three Asiatic *Castanea* species: 10 of *C. crenata* Sieb. e Zucc. (SS-CC), 10 of *C. mollissima* Blume (SS-CM) and 13 of *Castanea henryi* Rehd. & Wils. (SS-CH).
- Thirty-two accessions of *C. sativa* Mill. from three different seed sources: 12 accessions from the RP2 provenance region (Mountains and Plateaus of Galicia, north-western Spain), 10 accessions from the RP4 provenance region (Cantabric basin, northern Spain), and 10 accessions from the RP19 provenance region (Sierra de Gredos mountain range, Central Spain).
- Twenty accessions of *C. sativa* belonging to 12 clones of the following seven traditional fruit cultivars of Galicia: 'Blanca' (clones BL2B and BL4), 'Amarelante' (AM43), 'Garrida' (GA5 and GA37), 'Garrida de Chantada' (GACH), 'Parede' (PA27 and PA39), 'Luguesa' (LU18 and LU52) and 'Raigona' (RA6A and RA20).

In addition, a second set of 32 accessions from 15 trees was selected in the field based on their putative resistance to *Phytophthora* spp. They were tested to assign them as *C. sativa* or hybrids with Asiatic species based on molecular analysis. These trees were growing in coastal areas of Galicia and were coded C004, C005, C006, C011, C017, C036, C047, C048, P011, P013, P014 P015, P024, P035 and P042. To further enrich the molecular assignment, a third set of eight accessions from three clones of interspecific hybrids of *C. crenata* × *C. sativa* (clones HS, 111 and 2671) were included in the study.

Young leaves of Asiatic *Castanea* species and *C. sativa* from the forest provenances were harvested from certified seeds germinated in greenhouses at TRAGSA facilities (Maceda, Ourense, Spain). Young leaves of the fruit cultivars were obtained from selected trees vegetatively replicated by grafting and conserved in clonal orchards at TRAGSA facilities. Finally, young leaves of selected trees were harvested from acclimated plants obtained by *in vitro* propagation. All chestnut leaves were stored at −20 °C until use.

2.2. DNA extraction and simple sequence repeat (SSR) amplification

A genomic DNA extraction was carried out from 100 mg of leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

Twelve SSR loci were tested: KT002a, KT004a, KT005a and KT015a, isolated by Yamamoto et al. (2003) in *C. crenata*; CsCAT1, CsCAT3, CsCAT6, CsCAT14 and CsCAT16, developed by Marinoni et al. (2003) in *C. sativa*; EMCs14, developed by Buck et al. (2003) in *C. sativa*; and, finally, QpZAG36 and QpZAG110, developed by Steinkellner et al. (1997) in *Quercus petraea*.

PCRs for SSRs analysis were performed in a volume of 20 μl mixture containing reaction buffer (10 mM Tris–HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 25 ng of genomic DNA (except loci from Yamamoto et al., 2003, which were amplified from 10 ng) and 0.5 U of BIOTAQ DNA polymerase (Bioline, London, UK). Amplification reactions were performed in a thermocycler (Techne, Burlington, NJ, USA) using the appropriate temperature profiles for each primer. The KT002a, KT004a, KT005a and KT015a loci were amplified as described by Yamamoto et al. (2003), but by using 52.8, 57, 50 and 50 °C as annealing temperatures, respectively; the CsCAT1, CsCAT3, CsCAT6, CsCAT14 and CsCAT16 loci were amplified according to Marinoni et al. (2003); the QpZAG36 and QpZAG110 loci were amplified using the conditions described by Marinoni et al. (2003), with some modifications (initial step at 94 °C for 4 min and 30 cycles with 50 °C of annealing temperature); and the EMCs14 locus was amplified as described by Buck et al. (2003). Amplification reactions were carried out using the forward primer labeled with a fluorescent chemical (NED, FAM, VIC or PET) to enable the detection of

fragments on an ABI PRISM 3100 automatic DNA sequencer. Allele scoring was performed using the GeneMapper software (version 3.7, Applied Biosystems, Foster City, CA, USA).

2.3. Data analysis

To ensure the reproducibility of the data, one sample of *C. crenata* (SS-CC4) was always included in PCR amplifications as a control. SSR allele sizes were scored in bp. An analysis with the software Micro-Checker ver. 2.2.3 (Van Oosterhout et al., 2004) was performed to test the quality of allele scoring.

For every species and population (*C. crenata*, *C. mollissima*, *C. henryi*, *C. sativa* RP2, *C. sativa* RP4, *C. sativa* RP19 and *C. sativa* fruit cultivars), the probability of identity (*PI*, Paetkau et al., 1995), the observed and expected heterozygosity (*Ho* and *He*, Nei, 1973) as well as the frequency of null alleles (Brookfield, 1996) were determined using the software IDENTITY 1.0 (Wagner and Sefc, 1999). Allele frequencies and polymorphic information content (*PIC*) values were calculated using Microsatellite Tools for Excel (Park, 2001).

A phylogenetic tree of populations was constructed with DA genetic distance (Nei et al., 1983) and neighbour-joining method using the software Populations (Langella et al., 2001). The phylogenetic tree was visualized using the software TreeView (Page, 1996).

A factorial correspondence analysis (FCA) was applied on the SSR genotypes to graphically explore the distribution of genetic variation using the Genetix software (Belkhir et al., 1996).

Finally, an analysis of molecular variance (AMOVA, Excoffier et al., 1992) was calculated using Arlequin version 3.1 (Excoffier et al., 2005) to estimate the components of variance among species and within *C. sativa* populations.

3. Results

Nine out of twelve SSR markers were successfully amplified in the four *Castanea* species tested (KT015a, CsCAT1, CsCAT3, CsCAT6, CsCAT14, CsCAT16, EMCs14, QpZAG36, and QpZAG110). The remaining three loci (KT002a, KT004a, and KT005a) failed to generate reproducible fragments and were, hence, discarded for genotype identification.

With these nine SSR loci, a total of 172 alleles were detected in the 85 accessions of four species of *Castanea* tested in this part of the study. The average number of alleles per locus was 12. To avoid an estimation bias, a characterization of the nine loci was performed independently for every species and, in the case of *C. sativa*, for every population (Table 1). The cumulative probability of identity (*PI*) and the estimated frequency of null alleles were low (Table 1). Exceptions to this general rule were the null allele frequency of the CsCAT16 locus in *C. mollissima* and *C. henryi* as well as of the CsCAT14 locus in the RP4 and RP19 populations of *C. sativa*. However, an analysis conducted using Micro-Checker software showed that no significant deviations from Hardy–Weinberg equilibrium were detected for any species or population tested. Hence, all SSR data were used for further analysis.

The most polymorphic locus in the 3 Asiatic species was CsCAT3 (Table 1), showing 8–9 genotypes, 5–10 alleles (2–3 of them were privative, Table 2) and an observed heterozygosity (*Ho*) of 0.60–0.89. Similarly, in *C. mollissima*, the locus QpZAG110 showed a high variability, with 7 genotypes and alleles (without any privative one) and an observed *Ho* of 0.90. In *C. sativa*, the locus CsCAT6 showed, in general, a higher number of genotypes and alleles (including privative ones) than CsCAT3; this later locus showed good results, in particular, in the RP2 population. Likewise, CsCAT3 and CsCAT6 were the most informative loci (Table 1); CsCAT3 showed a polymorphic information content (PIC) ranging from 64 to 84% in Asiatic species, whereas CsCAT6 showed a PIC of 60–83% in *C. sativa* populations. In contrast, the least discriminating locus in all species was EMCs14, which was homozygotic in *C. crenata* and *C. mollissima* and produced only 2 alleles and genotypes in *C. henryi*; in *C. sativa*, it produced between 1 and 3 alleles and genotypes in all the tested populations. Moreover, the two privative alleles produced by this locus were found in *C. sativa* (Table 2).

The allelic frequencies of the nine SSR loci that amplified in both Asiatic species and *C. sativa* populations are shown in Fig. 1. Most of the SSR loci showed frequency distributions encompassing the entire range of lengths found in the four species tested. Nevertheless, there were four loci (CsCAT1, CsCAT3, CsCAT6, and CsCAT16) in which the distribution of *C. sativa* alleles (black range in Fig. 1) was essentially different from that of *C. mollisima* and *C. henryi* alleles (white range in Fig. 1), with only a few overlapping alleles.

Privative alleles were observed in the four analyzed species and, especially, for the five SSR described by Marinoni et al. (2003). Table 2 shows the privative alleles found in *Castanea* species and *C. sativa* populations. Four alleles, 186-CsCAT1, 210-CsCAT3, 136-CsCAT14 and 206-QpZAG36, were found in the three Asiatic species but never in *C. sativa*. Some of the privative alleles were frequent (Fig. 1), such as 132-CsCAT14 in *C. crenata* (40%), or alleles 190-KT015a (87%), 150-CsCAT6 (20%), 156-CsCAT16 (21%) and 186-CsCAT16 (28%) in *C. mollissima*. Frequent, privative alleles were also found in *C. henryi*, such as 188-KT015a (89%), 182-CsCAT1 (62%), 204-CsCAT3 (42%), 148-CsCAT6 (64%), 170-CsCAT16 (33%) and 212-QpZAG36 (29%). Two privative alleles (234-CsCAT3 and 134-CsCAT14) were found in all *C. sativa* populations, with frequencies ranging from 16% to 65%, while other nine privative alleles were present in more than one *C. sativa* population (Table 2). In addition, privative alleles of population were found in two *C. sativa* populations (RP2 and fruit accessions, Table 2). Out of them, only the allele 134-CsCAT16 (45.8%) in the fruit accessions population was significantly frequent.

Alleles shared exclusively by *C. sativa* accessions with the Asiatic species (17% of the total alleles) were also found (data not shown). Of them, 23 (79%) were shared with *C. crenata,* whereas only 2 alleles were shared with *C. mollissima* and 4 with *C. henryi*.

Once the framework of genetic variability in *Castanea* species and *C. sativa* populations was established, a phylogenetic tree based on DA genetic distance (Nei et al., 1983) was constructed for grouping all the tested chestnut populations using the neighbour-joining method. In this analysis, the *Castanea* plants selected by their putative resistance to *Phytophthora* spp. were also included. The tree showed two clearly distinct groups (Fig. 2), each including closely related species: *C. mollissima* and *C. henryi* on one side (Group I), and *C. sativa* (including the four populations) with *C. crenata* on the other (Group II). This later group also included all the selected plants that presented an intermediate position between those species.

Based on the structure of the phylogenetic tree, a factorial correspondence analysis (FCA) was applied on multilocus genotypes to graphically explore the distribution of genetic variation among accessions belonging to *C. crenata* and *C. sativa* to ascertain the putative genetic identity of the selected plants as hybrids or belonging to one of the tested *Castanea* species. Furthermore, three known hybrids of *C. crenata* × *C. sativa* (Cc × Cs2671, Cc × Cs111 and Cc × CsHS) were also included.

The FCA analysis was performed with the nine SSR data (Fig. 3) and showed 100% of variation accumulated in the first three axes; the first one explained over 51.82% of the total variance. A three-dimensional distribution showed the existence of two main groups, each corresponding to the Japanese and the European chestnuts; a third group corresponded to the known hybrid plants located at

Table 1
Characterization of the nine SSR loci tested in three chestnut Asiatic species and four populations of *C. sativa*.

Species/population	Locus	Allelic size range (bp)	Genotypes (*n*)	Alleles (*n*)	Frequency of null alleles	*Ho*[a]	*He*[b]	*PI*[c]	*PIC*[d]
C. crenata	KT015a	168–206	4	4	−0.182	1.00	0.69	0.28	0.63
	CsCAT1	186–222	7	5	0.007	0.67	0.68	0.22	0.64
	CsCAT3	198–262	8	10	−0.017	0.89	0.86	0.06	0.84
	CsCAT6	138–194	7	7	−0.066	0.89	0.77	0.15	0.74
	CsCAT14	132–160	6	5	0.099	0.50	0.67	0.29	0.60
	CsCAT16	130–148	4	4	0.081	0.33	0.45	0.39	0.42
	EMCs14	142	1	1	0.000	0.00	0.00	1.00	0.00
	QpZAG36	206–220	7	5	−0.095	0.90	0.73	0.51	0.69
	QpZAG110	208–218	5	4	−0.162	0.90	0.63	0.32	0.57
	Average			5	−0.04	0.68	0.61		
	Total			45				4.17×10^{-6}	
C. mollissima	KT015a	168–190	2	2	−0.026	0.25	0.22	0.68	0.19
	CsCAT1	178–186	5	4	0.077	0.33	0.44	0.42	0.41
	CsCAT3	206–216	8	5	0.053	0.60	0.69	0.24	0.64
	CsCAT6	134–150	4	4	0.016	0.50	0.53	0.36	0.48
	CsCAT14	136–150	3	4	0.000	0.44	0.44	0.42	0.41
	CsCAT16	156–186	5	5	0.353	0.14	0.77	0.17	0.73
	EMCs14	136	1	1	0.000	0.00	0.00	1.00	0.00
	QpZAG36	206	1	1	0.000	0.00	0.00	1.00	0.00
	QpZAG110	218–238	7	7	−0.067	0.90	0.78	0.14	0.75
	Average			3.67	0.05	0.35	0.42		
	Total			33				2.6×10^{-4}	
C. henryi	KT015a	168–188	3	3	−0.015	0.22	0.20	0.66	0.19
	CsCAT1	178–208	6	6	−0.002	0.58	0.58	0.25	0.56
	CsCAT3	202–222	9	7	−0.059	0.84	0.74	0.16	0.71
	CsCAT6	144–148	3	2	0.068	0.36	0.46	0.61	0.36
	CsCAT14	136–152	5	3	0.016	0.62	0.64	0.34	0.57
	CsCAT16	150–182	6	5	0.231	0.33	0.73	0.20	0.69
	EMCs14	136–139	2	2	−0.165	0.69	0.45	0.61	0.36
	QpZAG36	206–216	5	4	0.014	0.50	0.52	0.44	0.45
	QpZAG110	218–238	6	5	−0.128	0.85	0.64	0.27	0.59
	Average			4.11	−0.000	0.55	0.55		
	Total			37				8.2×10^{-5}	
C. sativa									
RP2	KT015a	168–196	4	3	−0.027	0.67	0.62	0.38	0.54
	CsCAT1	194–222	5	4	−0.029	0.58	0.54	0.38	0.48
	CsCAT3	224–248	7	8	−0.134	1.00	0.76	0.13	0.74
	CsCAT6	160–190	8	5	0.099	0.58	0.76	0.18	0.72
	CsCAT14	134–152	7	4	−0.039	0.75	0.68	0.25	0.63
	CsCAT16	132–150	5	6	−0.045	0.73	0.65	0.30	0.59
	EMCs14	132–150	3	3	−0.035	0.33	0.29	0.58	0.26
	QpZAG36	214–220	6	4	0.040	0.67	0.74	0.22	0.69
	QpZAG110	208–222	6	5	−0.017	0.73	0.70	0.22	0.65
	Average			4.67	−0.020	0.67	0.64		
	Total			42				7.4×10^{-6}	
C. sativa									
RP4	KT015a	168–196	2	3	−0.294	1.00	0.55	0.52	0.44
	CsCAT1	194–222	2	2	−0.007	0.13	0.12	0.80	0.11
	CsCAT3	198–234	4	4	−0.150	0.80	0.57	0.36	0.51
	CsCAT6	160–184	6	6	−0.159	0.90	0.64	0.24	0.60
	CsCAT14	134–150	3	2	0.189	0.20	0.48	0.62	0.36
	CsCAT16	132–146	5	3	0.100	0.40	0.56	0.39	0.49
	EMCs14	132–142	3	3	0.051	0.20	0.27	0.59	0.25
	QpZAG36	214–220	4	4	−0.165	0.88	0.61	0.30	0.56
	QpZAG110	208–226	4	4	0.073	0.40	0.51	0.41	0.45
	Average			3.44	−0.040	0.55	0.48		
	Total			31				6.3×10^{-4}	
RP19	KT015a	170–196	2	2	−0.036	0.30	0.26	0.65	0.22
	CsCAT1	194–222	5	3	−0.010	0.60	0.59	0.39	0.51
	CsCAT3	224–250	6	4	0.164	0.40	0.68	0.30	0.61
	CsCAT6	160–194	8	7	−0.041	0.90	0.83	0.10	0.80
	CsCAT14	134–152	5	3	0.215	0.30	0.66	0.34	0.58
	CsCAT16	132–146	3	3	−0.039	0.60	0.54	0.43	0.47
	EMCs14	142	1	1	0.000	0.00	0.00	1.00	0.00
	QpZAG36	214–220	6	4	−0.075	0.80	0.68	0.30	0.61
	QpZAG110	208–218	5	4	0.042	0.60	0.67	0.29	0.61
	Average			3.44	0.020	0.50	0.55		
	Total			31				9.5×10^{-5}	
C. sativa									
Galician traditional	KT015a	170–196	3	2	−0.182	0.77	0.50	0.62	0.37
fruit cultivars	CsCAT1	194–222	3	3	−0.030	0.54	0.49	0.46	0.43

Table 1 (*Continued*)

Species/population	Locus	Allelic size range (bp)	Genotypes (*n*)	Alleles (*n*)	Frequency of null alleles	*Ho*[a]	*He*[b]	*PI*[c]	*PIC*[d]
	CsCAT3	224–250	6	6	−0.058	0.85	0.75	0.18	0.71
	CsCAT6	160–182	10	9	0.005	0.85	0.86	0.07	0.83
	CsCAT14	134–152	5	4	−0.056	0.62	0.53	0.33	0.52
	CsCAT16	132–150	8	5	0.026	0.67	0.71	0.19	0.68
	EMCs14	132–142	2	2	−0.010	0.15	0.14	0.77	0.16
	QpZAG36	208–220	6	5	−0.101	0.85	0.68	0.22	0.60
	QpZAG110	208–222	4	5	−0.152	1.00	0.74	0.20	0.71
	Average			4.56	−0.060	0.70	0.60		
	Total			41				8.39×10^{-6}	

[a] *Ho*, observed heterozygosity.
[b] *He*, expected heterozygosity.
[c] *PI*, probability of identity.
[d] *PIC*, polymorphic information content.

Table 2
Privative alleles obtained with the nine SSR loci tested in three Asiatic species of *Castanea* and four populations of *C. sativa*.

	SSR loci								
	KT015a	CsCAT1	CsCAT3	CsCAT6	CsCAT14	CsCAT16	EMCs14	QpZAG36	Total
C. crenata	206	188, 214	260, 262	138	132, 160	130, 148			10
C. mollissima	190	184	212, 214, 216	134, 146, 150	148	156, 158, 186			12
C. henryi	182, 188	182, 192	204, 208, 220	148		170, 182		210, 212	12
Asiatic species[a]		186	210		136			206	4
C. sativa[b]		218	232, 234, 236, 248, 250	170	134	142, 144	132		11
C. sativa, RP2 population			228	190		136	150		4
C. sativa, Galician traditional fruit cultivars				164, 172		134		208	4
Total	4	7	15	9	5	11	2	4	57

[a] Alleles present in all Asiatic species tested but never in *C. sativa*.
[b] Alleles present in more than one *C. sativa* population.

an intermediate position between the species. Finally, the selected plants appeared at an intermediate position among all known populations but presented lower values than the hybrids in the third axis.

The AMOVA showed a larger proportion of variation among individuals than among *Castanea* species or *C. sativa* populations (Table 3). The largest contribution to variation among individuals was by loci QpZAG110 and CsCAT3, which explained about 86–89% of the variance (data not shown); moreover, loci CsCAT6 and QpZAG36 explained up to 74%. In contrast, locus EMCs14 explained only 33% of variance.

A detailed analysis of the SSR alleles showed that all of the selected plants, except C011, presented privative alleles from any of the chestnut species studied (Table 4). The selected plants coded P014, C017 and C004 presented privative alleles of *C. crenata*, *C. mollissima* and *C. sativa*, and remarkably, they showed two to three of the 4 alleles presented in the three Asiatic species but never in *C. sativa* (186-CsCAT1, 210-CsCAT3 and 206-QpZAG36). The three selected plants coded P011, P042 and P013 presented one privative allele of *C. crenata* (188-CsCAT1), one of *C. henryi* (204-CsCAT3) and two or three privative alleles of *C. sativa*. The selected plants P015 and P024 presented two privative alleles of *C. sativa*, but one of an Asiatic species (208 of CsCAT3 privative of *C. henryi* in P015, and 214-CsCAT1 privative of *C. crenata* in P024); in contrast, C006 presented 4 privative alleles of *C. sativa* as well as an allele (186-CsCAT1) absent in *C. sativa* but present in all the Asiatic species tested.

The remaining selected plants did not present privative alleles of any Asiatic species. In addition, C005 presented only one privative allele of *C. sativa* (134-CsCAT14), whereas P035, C047 presented 2 privative alleles of *C. sativa*. Finally, the selected plants coded C036 and C048 presented 5 privative alleles of *C. sativa*.

A special case was plant C011, which had all its alleles shared by *C. sativa* and *C. crenata*, and had no privative allele from Asiatic or European species.

4. Discussion

Nine SSR loci were useful to discriminate three Asiatic and the European species of *Castanea*. Four of the analyzed SSRs (KT002a, KT004a, KT005a, and KT015a) were described by Yamamoto et al. (2003) in *C. crenata* accessions. In our material, only the locus KT015a generated reproducible fragments, showing in *C. crenata* four alleles in a size range (168–206 bp) similar to that described by those authors (164–217 bp). Tanaka et al. (2005) showed that KT015a is a locus with few alleles in *C. crenata*, however, all their

Table 3
Analysis of molecular variance (AMOVA) of 85 accessions belonging to Asiatic (*C. crenata*, *C. mollissima*, *C. henryi*) and European (*C. sativa*) chestnuts based on the nine SSR loci.

Source of variation	Sum of squares	Variance components	Percentage variation	Fixation indices	*P* value
Among species	141.059	0.73723	19.31048	0.19310	0.00000
Among populations					
Within species	25.414	0.23759	6.22327	0.07713	0.00000
Among individuals					
Within populations	274.001	0.30649	8.02792	0.10781	0.00000
Within individuals	243.500	2.53646	66.43833	0.33562	0.00000
Total	683.974	3.81776			

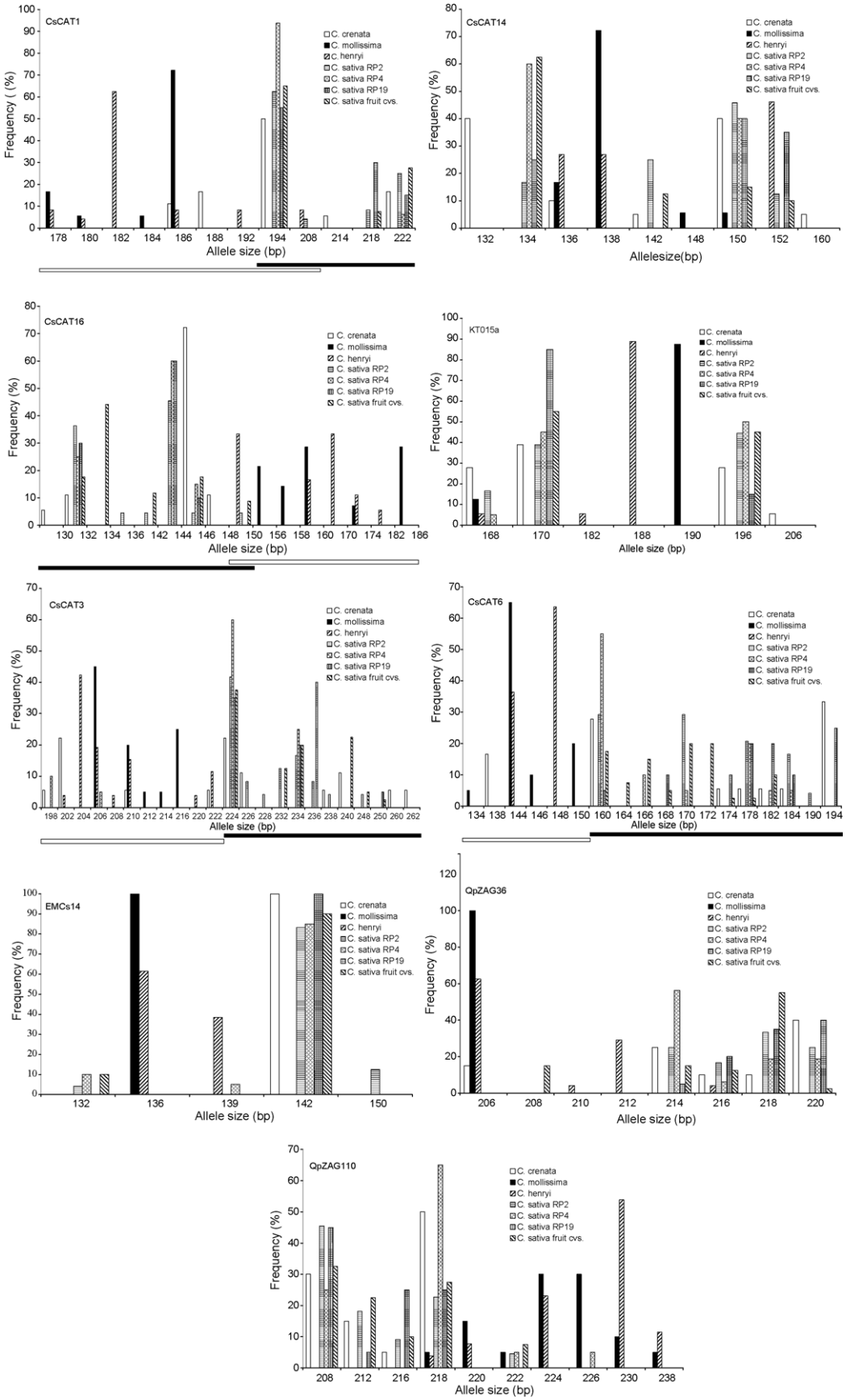

Fig. 1. Alleles and related frequencies found for nine SSR loci in 85 accessions of three Asiatic *Castanea* species (*C. crenata*, *C. mollissima*, and *C. henryi*) and four populations of the European chestnut (*C. sativa*). Horizontal boxes below the *X* axis in the figures of CsCAT1, CsCAT3, CsCAT6, and CsCAT16 loci indicate the allelic range found in *C. mollisima* and *C. henry* accessions (white box) and in *C. sativa* accessions (black box).

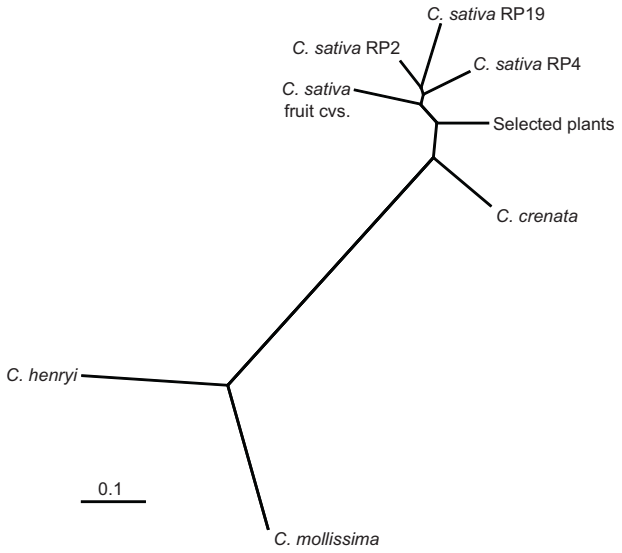

Fig. 2. Unrooted phylogenetic tree of three Asiatic *Castanea* species (*C. crenata*, *C. mollissima*, and *C. henryi*), four populations of European chestnut (*C. sativa*) and the selected plants. The tree was constructed by the neighbour-joining method using the software Populations on the basis of nine SSR loci.

major alleles with high frequencies were distributed in a narrow size range (162–168 bp). In addition, Yamamoto et al. (2003) tested this locus in other chestnut species and obtained similar results in *C. sativa* to those obtained here.

Likewise, CsCAT3 and CsCAT6 have already been described as loci with a high allele number in Italian cultivars of *C. sativa* (Marinoni et al., 2003). We found a high polymorphism for these loci, which allowed us to detect privative alleles in the four analyzed species. In addition, CsCAT3 and CsCAT6 showed a size range in *C. sativa* clearly different from *C. mollissima* and *C. henryi*, withno shared alleles (Fig. 1). These two loci were successfully used by Martin et al. (2009) for the identification of traditional chestnut varieties from southern Spain. Locus CsCAT3 was the most informative (Table 1) and, together with the QpZAG110 locus, explained the largest proportion of variance among individuals in the AMOVA analysis (see Section 3). In contrast, the least useful locus seemed to be EMCs14, which presented the lowest number of alleles and genotypes (Table 1), explaining the lowest proportion of variance among individuals in the AMOVA analysis.

Moreover, CsCAT16 was another polymorphic locus (16 alleles in total), with an allele size range in *C. sativa* different from *C. mollissima* and *C. henryi*. However, there is a shared allele (150-CsCAT16) with the latter species (Fig. 1). In addition, this locus produced a high number of privative alleles (11 alleles, Table 2); however, it presented null alleles in *C. mollissima* (0.353) and *C. henryi* (0.231), which is in agreement with results recently obtained by Pereira-Lorenzo et al. (2010) in the same Asiatic species. Yet, this locus was in Hardy-Weinberg equilibrium as revealed by an analysis using Micro-Checker software (see Section 3).

A different allele size range in *C. sativa* vs. *C. mollissima* and *C. henryi* was also observed for the locus CsCAT1 (Fig. 1). However, this locus was not as discriminant as the aforementioned ones because it presented two shared alleles (194-CsCAT1, 208-CsCAT1). No differentiating allele size range was observed between *C. sativa* and *C. crenata* in any locus.

A higher number of privative alleles in Asiatic species (22% of the total alleles) than in *C. sativa* (11%) were found (Table 2). Pereira-Lorenzo et al. (2010) found a much higher proportion of privative alleles in the same Asiatic species, stating that this may indicate a high degree of diversity in them than in *C. sativa*. In contrast, most of the alleles shared exclusively by *C. sativa* accessions with the Asiatic species (see Section 3) were with *C. crenata*. This result agrees with those of Pereira-Lorenzo et al. (2010), and it may explain the success of the introduction of *C. crenata* in Spain as part of hybridization programs aimed at developing hybrids resistant to ink disease at the early 20th century (Gallastegui, 1926; Urquijo, 1944).

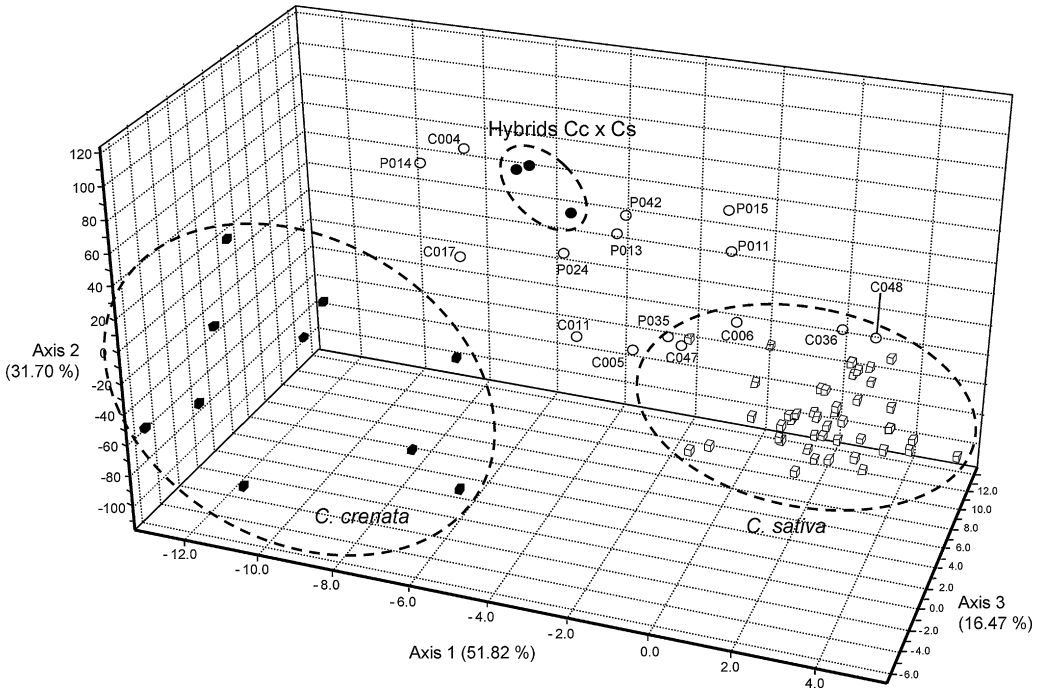

Fig. 3. Three-dimensional factorial correspondence analysis based on SSR data showing chestnut accessions corresponding to *Castanea crenata* (black cubes), *C. sativa* (white cubes), *C. crenata* × *C. sativa* hybrids (black spheres) and chestnut plants selected for their putative resistance to the ink disease (white spheres, each plant marked with its code).

Table 4
SSR genetic profiles of 15 *Castanea* plants selected for their resistance to *Phytophthora* spp. Privative alleles of the Asiatic *Castanea* species are underlined. Privative alleles of *C. sativa* are in bold. Allele sizes are given in base pairs. NA: not amplified.

Selected plant	SSR loci								
	KT015a	CsCAT1	CsCAT3	CsCAT6	CsCAT14	CsCAT16	EMCs14	QpZAG36	QpZAG110
C004	168:170	186:194	202:226	156:**170**	142:150	**136**	142	206:218	208:218
C005	170	194	224:226	160:174	**134**	146	142	214:218	218
C006	170	186:194	226:**236**	160:**170**	**134**	**144**	142	214:218	218
C011	170	194	198:226	160:178	142	146	142	214:220	218
C017	168:196	194	210:**234**	138:160	**134**	146:150	142	206:214	218:220
C036	170:196	216:**218**	**236:250**	160:166	**134**:150	**134**:150	142	214:218	216:222
C047	170:196	194	198:**234**	184	**134**	146	142	216	208:212
C048	170:196	194	**234**	**170**	**134**:142	**144**	**132**:142	218	212:218
P011	170	188:194	**232**:204	166:174	**134**	**144**	142	218	216:220
P013	168:170	188:194	204:238	156:166	142:150	**144**	142	**208**:218	208:216
P014	160:170	186:194	210:**234**	138:174	**134**:148	150	142	206:218	208:212
P015	NA	194	208:244	174:184	**134**:142	**134**:150	142	216:218	212:218
P024	170	194:214	198:244	**170**	**134**:142	146	142	218	212:218
P035	170:196	194	226:**234**	160:184	**134**:142	146:150	142	214:216	218
P042	168:196	188:194	204:224	156:166	**134**:150	150	142	**208**:218	208

The joint information provided by the FCA analysis (Fig. 3) and the presence of privative alleles (Table 4) allowed the putative molecular assignment of identity of the selected plants as *C. sativa* or hybrids. Thus, three selected plants (C004, P014 and C017) were located among the known hybrids and *C. crenata* accessions in the FCA graphic; moreover, they had privative alleles of the tested Asiatic species. Hence, these plants were assigned as hybrids, where *C. sativa* could be one of the parentals due to the presence of some privative alleles of the European chestnut.

All the remaining selected plants appeared between the known hybrids and *C. sativa* accessions in the FCA graphic (Fig. 3). From these, a group of five (P011, P013, P015, P024, P042) shared privative alleles of both *C. sativa* and Asiatic species (Table 4), suggesting that they probably were hybrids of uncertain origin, but with *C. sativa* as one of the parentals.

The finding of hybrid plants among the selected plants is interesting because this hybrid condition was the most probable source of resistance to *Phytophthora*. In addition, some of these plants had a strong genetic component of *C. sativa* (mainly P011, P015 and P024 plants); therefore, they could present high compatibility with *C. sativa*, representing a good source of rootstocks for grafting. This was already described for the HS hybrid (Pereira-Lorenzo et al., 2010), the most utilized rootstock in Spain (Pereira-Lorenzo and Fernández-López, 1997).

The plant coded C011 did not present any privative allele of any species, but it had a number of alleles shared by *C. crenata* and *C. sativa* accessions. Hence, C011 could be regarded as a hybrid between those two species. The position of the plant coded C006 in the FCA graphic (Fig. 3) and the presence of four privative alleles of *C. sativa* (Table 4) suggest that it belongs to this species. However, it also presented a privative allele of Asiatic species (186-CsCAT1, Table 2).

A particular group of plants presented one (C005) or two (C047 and P035) privative alleles of *C. sativa*, and none of Asiatic species. They appeared close to a *C. sativa* accession (CsRP2-6) in the three-dimensional graphic (Fig. 3). Moreover, they shared with it a number of alleles (7–9 out of 12–15) and had 1–2 privative alleles of *C. sativa* (data not shown). Hence, these plants could be considered as *C. sativa*; however, their low number of privative alleles from this species produces some degree of uncertainty about this assignment.

Finally, plants coded C036 and C048 did not present any privative allele of Asiatic species, but they presented up to five privative alleles of *C. sativa*. These outcomes and their position close to the *C. sativa* cluster in the three-dimensional graphic (Fig. 3) strongly support their assignment to a *C. sativa* genotype.

5. Conclusion

To conclude, we found nine SSR markers useful to distinguish Asiatic *Castanea* species from the European one (*C. sativa*). These markers were also useful to assign unknown plants selected by their resistance to *Phytophthora* spp. to hybrids or *C. sativa* genotypes. Thus, as some of these selected plants assigned to hybrids had a strong genetic component of *C. sativa* they could be very interesting as rootstocks for grafting. Other two selected plants have been most probably assigned to *C. sativa*, hence being good candidates for their inclusion in the Spanish National Catalogue of Forest Base Materials with commercialization purposes.

Acknowledgments

We gratefully acknowledge Drs. Román Vilas and Jaime Castro (Departamento de Genética, Universidad de Santiago de Compostela, Spain) for their help with the analyses of the SSR data. We also thank María L. Villar from the same department for her technical support with fragment analysis.

We are grateful to Juan José Miguens, Alejandra Santás, Belén Caride and Begoña Lueiro for their excellent technical assistance in different stages of this study.

This paper is a contribution of the Interuniversity Research Group in Biotechnology and Reproductive Biology of Woody Plants (BioVitAc research group, code 09IDI1705). This research was supported by the Xunta de Galicia (project PGIDIT07MRU003E, Regional Government of Galicia, Spain).

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