Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/forestsystems

Forest Systems 2011 20(1), 65-80
ISSN: 1131-7965
eISSN: 2171-9845

Identification of the genealogy of interspecific hybrids between *Castanea sativa, Castanea crenata* and *Castanea mollissima*

J. Fernandez-Lopez*

Centro de Investigación Forestal de Lourizán. Xunta de Galicia. Ctra. de Marín, km 4. 36080 Pontevedra. Spain

Abstract

The massive mortality of *Castanea sativa* in southwestern Europe, which was caused by different species of *Phytophthora* spp., led to the introduction of seeds of the Asiatic species *Castanea crenata* and *Castanea mollissima* and to hybridization to breed for resistance to *Phytophthora* spp. In Spain, two programmes were developed: one programme, focussed mainly on obtaining first generation hybrids by controlled pollinations, and the other programme, based on selection among open-pollinated progenies collected from first and second generation hybrids, in both cases between sweet and Japanese chestnut. A clone collection of 194 of the clones obtained is conserved at the Lourizán Forest Reseach Centre, and 32 of these were approved as basic material for forest reproduction. A sample of 356 individuals was genotyped using 13 isozyme *loci*, including the clone collection and several stands of Asiatic species. Only three *loci* were identified as being diagnostic among these species. The diversity of stands of both Asiatic species was reduced compared to that of *C. sativa*. Genotype inspection of diagnostic *loci* and two Bayesian procedures (*STRUCTURE* and *NEWHYBRIDS*) were used to classify all individuals into genealogical classes and, thus, reconstruct the history of chestnut hybridization in Spain.

Key words: chestnut; diagnostic *loci*; genealogic class; genotypic class; glandular trichomes; isozyme.

Resumen

Identificación de la genealogía de los híbridos interespecíficos entre *Castanea sativa, Castanea crenata* y *Castanea mollisima*

La elevada mortalidad de la especie *Castanea sativa* en el Suroeste de Europa, causada por diferentes especies del género *Phytophthora* spp. llevó a la introducción de semillas de las especies asiáticas *Castanea crenata* y *Castanea mollissima* y posteriormente a la hibridación para realizar mejora por resistencia a *Phytophthora* spp. En España se desarrollaron dos programas de mejora: uno de ellos basado en la obtención de híbridos de primera generación obtenidos por polinización controlada y otro basado en la selección dentro de progenies de polinización abierta de híbridos de primera y segunda generación, en ambos casos entre *C. crenata* y *C. sativa*. El Centro de Investigación de Lourizán conserva un núcleo de propagación con 194 clones procedentes de ambas selecciones y 32 de estos clones fueron aprobados como materiales de base forestales. Una muestra de 356 individuos se genotipó con 13 *loci* isoenzimáticos. Solo tres *loci* se identificaron como diagnóstico entre especies. La diversidad de los rodales de especies asiáticas resultó reducida en comparación con la diversidad de *C. sativa*. La inspección de *loci* diagnóstico y dos procedimientos bayesianos (*STRUCTURE* y *NEWHYBRIDS*) se utilizaron para clasificar los individuos en clases genealógicas y así reconstruir la historia de la hibridación de castaño en España.

Palabras clave: castaño; clase genealógica; clase genotípica; *loci* diagnóstico; tricomas glandulares; isoenzimas.

Introduction

The massive mortality of sweet chestnut (*Castanea sativa* Miller) in southwestern Europe due to damage caused by different species of *Phytophthora*, which is the causal agent of «ink disease» and results in rotting of root systems, led to the adoption of different measures to combat ink disease during the first half of the twentieth century. One measure taken was the introduction of Asiatic chestnut germplasm, which is considered to be resistant to the disease. This was proposed in France by Prunet in 1908 (Camus, 1929), and during subsequent decades, seeds of Japanese and Chinese chestnuts (*Castanea crenata* Sieb. et Zucc. and *Castanea mollissima* Blume, respectively) were introduced

* Corresponding author: josefa.fernandez.lopez@xunta.es
Received: 22-04-10; Accepted: 03-02-11.

to several southern European countries. The introduction of these species in Vizcaya (north Spain) took place between 1917 and 1940 (Elorrieta, 1949), and after 1940, they were introduced to Galicia from Vizcaya. Very soon afterwards, the low value of the Asiatic species as wood producers was recognised because their vigour is lower than that of sweet chestnuts, and they exhibit a lack of compatibility for grafting with local sweet chestnut varieties and a lack of adaptability to drought (Camus, 1929; Elorrieta, 1949).

Chestnut hybridisation was initiated in France by Couderc (Camus, 1929) and in Spain by Gallástegui (1926), who developed controlled crosses between *C. sativa* and *C. crenata*, obtaining a reduced number of first and second generation hybrids. By 1940, different breeding programmes had been developed in Spain (Urquijo Landaluze, 1957; Vieitez, 1966; Molina and Vieitez, 1967), France (Schad *et al*., 1952; Salesses *et al*., 1993), Switzerland (Bazzigher, 1981) and Portugal (Taveira-Fernándes, 1972). The objectives of these programmes were to breed for resistance to ink disease and, in Switzerland, to chestnut blight [*Chryphonectria parasitica* (Murrill) Barr], as well as to produce rootstock or varieties selected for early nut production or wood production and for better adaptation to drought than is exhibited by the Asiatic species. Clonal plantations of hybrids for wood and nut production have been developed in northwestern Spain, France and Portugal during the last five decades using selected hybrids.

In Galicia (northwestern Spain), two programmes were developed, one of which was developed between 1939 and 1958 at the Estación de Fitopatología Agraria de la Coruña (*EFAC*), while the other was developed under the Misión Biológica of Pontevedra (*MB*). The work at *EFAC* (Urquijo, 1944, 1957) consisted of obtaining hybrids by controlled pollinations using as mother plants 47 *C. sativa* trees from around the city of Coruña, which were crossed with ten pollinators, nine of which were *C. crenata*, while one was *C. mollissima*; additionally, some F_3 plants were obtained from open-pollinated progenies of F_2 individuals between *C. crenata* and *C. sativa* obtained by Gallástegui; finally, some natural hybrids were obtained in Córgomo-Valdeorras (for example clone 90044). The plants were tested for resistance to *Phytophthora* by inoculation and based on their survival in infected soils. The *MB* programme (Vieitez, 1966) consisted of the inoculation of open-pollinated progenies collected from: 3 first and 15 second generation hybrids between *C. crenata* and *C. sativa* obtained in 1922 by Gallástegui (1926); 6 natural hybrids; 9 *C. sativa* trees; and 5 *C. crenata* trees. In 1956, all of the selections from the *MB* and a reduced number of selections from the *EFAC* were transferred to the Lourizán Research Centre for their propagation and selection for agronomic and forest traits.

In 1989, the Lourizán Research Centre initiated a programme for the identification, clonal propagation and selection of clones resistant to *Phytophthora* spp., with wood production as the main objective. A clone collection of 179 interspecific Euroasiatic hybrids that had been selected during the 1950s and 1960s in Spain and a number of *INRA* hybrids was established during the period from 1994-1997 to provide mother plants for vegetative propagation (Fernández-López *et al.*, 1995). The genealogy of each clone was unknown, with the exception of the *EFAC* and *INRA* clones. Several tools that are useful for clonal forestry, such as the identification of polymorphic isozyme loci for clone identification (Fernández-López *et al*., 1995), clonal propagation methods (Fernández-López *et al.,* 1992; Miranda Fontaiña and Fernández-López, 2001), characterisation for resistance to *Phytophthora* spp. (Fernández-López *et al*., 2001; Miranda-Fontaiña *et al*., 2007) and evaluation methods for vigour and stem form (Fernández López *et al*., 2008), were developed. Thirty-two clones were approved as base material for wood production (Boletín Oficial del Estado, 2007).

Identifying individuals of the sweet chestnut (*C. sativa*), the only European chestnut species, as well as of the Japanese (*C. crenata*) and Chinese (*C. mollissima*) chestnuts by use of different morphological, anatomical or phenological traits is usually feasible for botanists. Various species identification keys have been produced by different authors (Camus, 1929; Graves, 1949). The most useful trait for the identification of pure Japanese chestnut individuals is the flower-shaped glandular trichomes that are present on the abaxial surface of the leaves until senescence (Fig. 1a) (Camus, 1929; Graves, 1949). The presence of very long, abundant and solitary unicellular trichomes (> 1 mm) along the leaf midrib and in 1-year-old stems usually allows identification of Chinese chestnut individuals (Fig. 1b). The width, colour and transverse section of young shoots, as well as the leaf form, its aspect and size, male catkin stiffness and phenology are also useful for species identification.

Difficulties in identifying individuals increase when interspecific hybrids of different genealogical classes are present, as is usually the case in southwestern European countries where the Asiatic germplasm has

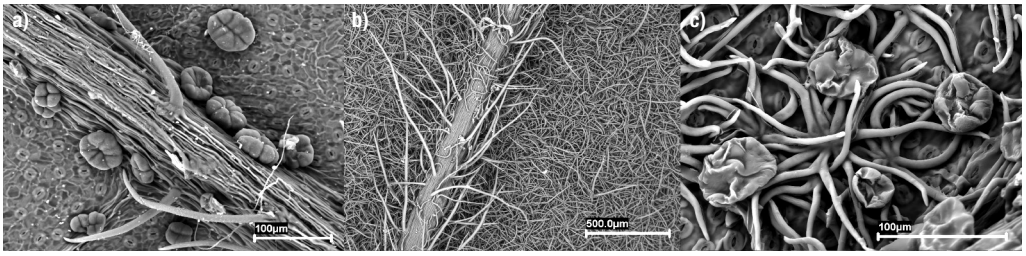

Figure 1. Scanning electron microscope images showing micromorphological features of the abaxial foliar surface, including some morphological traits used for identification: a) Japanese chestnut from the stand Prado das Troitas with perfect type glandular multicellular sessile trichomes (600X); b) Chinese chestnut *MB-13* with long unicellular trichomes along midrib (350X); c) collapsed type glandular trichomes in clone X, a hybrid of *Castanea crenata* × *C. sativa* (1000X), probably a backcross to *C. crenata*. The images were done in the Unidade de Microscopia Electrónica (*CACTUS*), University of Santiago de Compostela.

been cultivated since the second decade of the twentieth century. Natural hybridisation between individuals of sweet and Japanese chestnuts and between their hybrids occurs at a high frequency where the germplasm of both species coexists due to overlapping of the flowering period, as the species are entirely compatible with regard to pollinisation and fertilisation (Jaynes, 1964).

Different authors investigating hybridisation events in chestnut species in America and Europe have selected different traits to confirm the identities of putative interspecific hybrids (Urquijo-Landaluze, 1944; Graves, 1949; Schad *et al*., 1952). In particular, the distinction between the sweet chestnut and *Castanea crenata* × *C. sativa* hybrids usually presents problems. F_1 hybrids frequently have similar traits to the European chestnut, such as in the form and width of the shoot section, as well as the leaf form and length. The presence of collapsed glandular trichomes was also noted on the abaxial surface of the leaves of Euro-Japanese hybrids (Fig. 1c) (Jaynes, 1964). Identification of interspecific hybrids between different chestnut species by use of phenotypic traits is, therefore, difficult.

Genetic markers may be useful for the identification of species and hybrids that are similar in morphology when they are divergent at one or several *loci*. Different degrees of divergence between two species at a locus can be identified including: i) divergence consisting of fixation for different alleles in the two different species, in which case the locus is considered diagnostic for species and hybrid identification; ii) alleles that are not fixed within one species but are exclusive to the species; and iii) alleles common to both species, but with different allelic frequencies. The genealogical classes, *i.e.,* different degrees of relatedness between individuals, that are usually identified between two hybridising species are: two parental species, first and second generation hybrids and backcrosses of hybrids to each parental species (Nasson and Ellstrand, 1993). A single diagnostic *locus* is sufficient to discriminate between parental and F_1 genotypic categories in the absence of backcrosses, F_2 individuals and matings beyond F_2, and more *loci* are needed when the two last genealogical classes are present (Epifanio and Philipp, 1997; Boecklen and Howard, 1997). During the last decade, two different Bayesian approaches have been used for identifying hybrids that do not require diagnostic *loci*: *STRUCTURE* (Pritchard *et al.*, 2000), which clusters individuals into a number of *K* populations, while assuming that individuals within each population are in Hardy-Weinberg and linkage equilibrium, is applied for the identification of distinct genetic populations and admixed individuals; *NEWHYBRIDS* (Anderson and Thompson, 2002) calculates the probability of belonging to each of the different genealogical classes that are possible between two species for each individual. A comparison between the two methods indicated their high efficiencies and a better performance for the *NEWHYBRIDS* programme (Vähä and Primmer, 2006) when the objective is the assignation of individuals to genealogic classes.

The identification of the genealogical origin of individuals included in the nuclear stock of chestnut hybrids is useful for clones that are selected within the collection for descriptive purposes and for selection of individuals for the progressive breeding of these species. The identification of genealogical classes is also relevant in the conservation of autochthonous populations of sweet chestnut in areas where the planting of hybrids is intensive and introgression is expected. The presence of backcrosses to *C. sativa* is expected

in the local chestnut populations within the Atlantic area of the Iberian Peninsula, where the planting of hybrid clones for wood production is recommended.

The objectives of the present study were: 1) to describe the diversity of the Asiatic chestnut species *C. crenata* and *C. mollissima*, which have been introduced in Spain, and 2) to classify clones included in the collection of the Lourizán Forest Research Centre into genealogical classes using polymorphic isozyme loci and three sets of reference samples of pure *C. sativa*, *C. crenata* and *C. mollissima* through the genealogical inspection method, as well as using the *STRUCTURE* and *NEWHYBRIDS* software platforms. Although we know that this small number of markers is not enough for the identification of advanced generation hybrids and backcrosses, we expect to achieve a good level of identification of the species involved in hybridisation events and to produce several hybrid indices that will be useful for understanding the results of field clone tests and tests of resistance to *Phytophthora* spp.

Material and methods

Plant material

The total list of sampled materials is composed of 356 individuals (Table 1). The sweet chestnut was represented by 45 individuals, 36 of which were collected from two wild populations in northwestern Spain and nine of which were clones of Romanian origin. Several plantations of Asiatic species were identified in the Lourizán arboretum (stands Granxa da Serra, Prado das Troitas, As Minas and Agrovello and the Molina plantation line) and in the *MB* garden. Both research centres are located in Pontevedra (Galicia, NW Spain). The Granxa da Serra, Prado das Troitas e As Minas stands were planted in or before 1945, at the end of the period of Asiatic species introduction, and were registered as Japanese chestnuts. The Granxa da Serra stand was registered as '*Castanea koraiensis*', which is considered to be a *C. crenata* subtype from Korea. The Agrovello stand was registered as Chinese chestnuts. The number of trees selected was 15, 21, 17 and 21 in the Granxa da Serra, Prado das Troitas, As Minas and Agrovello stands, respectively. The trees in the Fernando Molina plantation line were planted in 1947 and are known to include four Japanese chestnuts *M-3*, *M-6*, *M-9* and *M-15*), four sweet chestnuts (*M-1*, *M-4*, *M-7*, *M-10*), three first generation hybrids between *C. crenata* and *C. mollissima* (*M-2*, *M-5*, *M-11*) and one Chinese chestnut (*M-14*). The Gallástegui plantation line in the *MB* is composed of 27 trees, including some Chinese and Japanese chestnut trees and interspecific hybrids, some of which were probably planted by Gallástegui, the first Spanish chestnut

Table 1. Plant material sampled in different stands, plantation lines and clone collection. Individuals of pure species, identified by use of several morphological traits, were used as reference samples

Population	Location	Expected genotypes[2]	No. trees	Reference samples			Unknown genealogy
				Cs	*Cc*	*Cm*	
Wild Galician	Galicia	*Cs*	36	36			0
Romanian cl.	Lourizán C[1]	*Cs*	9	9			0
Granxa da Serra	Lourizán	*Cc*	15		7		8
Prado das Troitas	Lourizán	*Cc*	21		17		4
As Minas	Lourizán	*Cc*	17		13		4
Agrovello	Lourizán	*Cm*	21			15	6
Fernando Molina	Lourizán	*Cs, Cc, Cm, F*$_1$(*CcxCs*)	12			1	0
Misión Biológica	Salcedo	Several	27			7	20
INRA	Bordeaux	*Cm*	4			4	0
MB + *EFAC*[3] clone	Lourizán C[1]	Hybrids dif. *GC*	173				173
INRA[3] clone	Lourizán C[1]	*F*$_1$(*CcxCs*)	6				0
EFAC[3] clone	Lourizán C[1]	*F*$_1$(*CcxCs*) mainly	15				15
Total			356	45	37	27	230

[1] *C*: Clone Collection with three ramets of each clone obtained from one single ortet. [2] *Cs*: *Castanea sativa; Cc: Castanea crenata; Cm: Castanea mollissima; F*$_1$(*CcxCs*): first generation hybrids between *C. sativa* and *C. crenata;* dif. *GC:* different genealogical classes. [3] *MB:* Misión Biológica; *EFAC:* Estación de Fitopatología Agraria de la Coruña; *INRA:* Institute Nationel de la Recherche Agrarie.

breeder. Four additional samples of Chinese chestnuts, *G-1-M*, *G-I-MI6*, *578* and *738*, that were collected in the *INRA* Bordeaux arboretum were also included.

Another 194 clones from the Clone Collection of chestnuts at the Lourizán Research Centre, most of which have unknown genealogy, were included in the study. Their origin can be classified into three groups:

Most clones (173) included in the nuclear stock had been in Lourizán since 1958. The majority of these probably originated in the *MB* programme, but a maximum number of 22 clones could have originated in the *EFAC* programme. Previous data on the *MB* clones indicate that they are progenies from a maximum of 24 open-pollinated progeny from F_1 and F_2 hybrids of *C. crenata* x *C. sativa* (Vieitez, 1985).

Six French F_1 hybrids of *C. crenata* × *C. sativa* chestnuts (Schad *et al.,* 1952) sent by *INRA* were also included in the nuclear stock.

An additional fifty clones from the disappeared *EFAC* were introduced to the Lourizán nuclear stock of chestnut hybrids in 1997. Most of these were F_1 individual from controlled crosses with *C. crenata* or *C. mollissima* as the male parent and *C. sativa* as the female parent, and a few hybrids were obtained from open pollinations of *C. sativa* trees or from F_2 individuals from crosses between the sweet chestnut and Japanese chestnut (Urquijo-Landaluce, 1944; 1957).

Morphological identification

The main objective of morphological identification was identifying pure individuals of each species. Individuals expected to belong to a pure species (reference samples in Table 1) were identified as belonging to the species by the use of morphological traits of current year shoot and leaves, as noted in the Introduction. Of particular interest were the glandular trichomes that were observed with an optical microscope at a magnification between 200X and 400X. Several types of glandular trichomes are present on the abaxial surface of *C. sativa* and *C. crenata* (Camus, 1929; Graves, 1949; Rinallo and Bruno, 1991). 'Perfect' glandular trichomes that are typical of *C. crenata* have a high taxonomic value (Graves, 1949) because of their different morphology and persistence. They are sessile, multicellular, of 40-80 μm in diameter, and have four to eight petal-like cells present throughout the abaxial leaf surface (Fig. 1a). 'Collapsed' glandular trichomes (Jaynes, 1964) that are typical of *C. crenata* × *C. sativa* hybrids can be identified with a scanning electron microscope (Fig. 1c). Their identification with an optical microscope is also easy in some cases and difficult in others. The presence of the perfect type of glandular trichomes, the collapsed type or their absence was determined in 141 individuals from the Lourizán clone collection using an optical microscope.

Laboratory methods

The methods used were previously described in Fernández-López and Monteagudo (2010). Two additional types of gels were used in this study: morpholine-citrate *pH* 8.1 ($MC_{8.1}$), and tris-citrate *pH* 8.8 ($TC_{8.8}$), as described by Wendel and Weedel (1989). Two controls were used for band identification: one European chestnut tree and one Japanese chestnut tree, which were identified as *M-1* and *M-3*, respectively. Gels were run at a constant intensity of 35 *mA* for three hours for morpholine, and for seven hours for tris-citrate. The isozymes assayed were: aconitase (*Aco**; *E.C.*4.2.1.3), phosphoglucoisomerase (*Pgi**; *E.C.* 5.3.1.9) and phosphoglucomutase (*Pgm**; *E.C.*2.7.5.1) on histidine gels, malate dehydrogenase (*Mdh**; *E.C.*1.1.1.37) on histidine-citrate gels, shikimate dehydrogenase (*Sdh**; *E.C.*1.1.1.25) and isocitrate dehydrogenase (*Idh**; *E.C.*1.1.1.42) on morpholine-citrate 6.1 gels, and phosphogluconate dehydrogenase (*Pgd**; *E.C.*1.1.1.44), diaphorase (Dia; E.C. 1.6.4.3), UTP-glucose-1-phosphate-uridyltransferase (*Ugp*; *E.C.* 2.7.7.9), peroxidase (*Prx*; *E.C.* 1.11.1.7) and fructose biphosphate (*Fdp*; *E.C.* 3.1.3.11) on morpholine-citrate 8.1 gels. Designation of loci when more than one locus was present was in the direction from anode to cathode as *Pgi-1*, *Pgi-2*, etc.

All 341 individuals were genotyped for loci within the cited enzyme systems, but 15 additional clones from *EFAC* were genotyped only for loci resolved in the seven enzyme systems marked with an * in the preceding paragraph.

Data analysis

Individuals of each pure species were selected to act as reference samples after considering their diagnostic morphological traits (Table 1): 45 *C. sativa*, 37 *C. crenata* and 27 *C. mollissima*. Zymograms were then produced for each species, and the level of polymorphism, computed on the basis of a 5% criterion, expected

heterozygosity, *Fst* between pairs of species and the allelic frequencies of each species were estimated with *GENALEX* (Peakall and Smouse, 2006). Diagnostic *loci* were determined, *i.e.,* alleles that were fixed for some species, as well as exclusive alleles, *i.e.,* species-specific alleles that are not fixed within one species.

The genealogical classes, i.*e.,* different degrees of relationship between individuals that were possibly present in the collection were the six basic classes that are possible for the group of *C. sativa* and *C. crenata.* Only two additional classes involving *C. mollissima* were expected, considering the scarce use of this species in the Spanish breeding programs (one parent among 10 used in controlled crosses in the *EFAC* programme) and the low success of crosses between *C. mollissima* and *C. sativa* (Jaynes, 1964). The eight genealogical classes (Table 2) were pure *C. sativa* (*Cs*), pure *C. crenata* (*Cc*), pure *C. mollissima* (*Cm*), first generation hybrids of *C. crenata* × *C. sativa* [F_1(*CcxCs*)], first generation hybrids of *C. mollissima* × *C. sativa* [F_1(*CmxCs*)], second generation hybrids of *C. crenata* × *C. sativa* [F_2(*CcxCs*)], backcrosses of [F_1(*CcxCs*)] on *Cc,* [*BC*(*Cc*)] and backcrosses of [F_1(*CcxCs*)] on *Cs,* [*BC*(*Cs*)]. According to published data, it was expected that most individuals included in the clone collection were F_1(*CcxCs*) for the *EFAC* and *INRA* clones and F_2(*CcxCs*) or F_3(*CcxCs*) for the *MB* clones.

The identification of the genealogical class to which each individual belonged was conducted using three methods: genotype inspection of diagnostic *loci* and the methods implemented in the software packages *STRUCTURE* and *NEWHYBRIDS*.

Table 2. The genealogical classes expected within the studied material are the six elemental genealogical classes in the *Castanea sativa* and *Castanea crenata* complex and two genealogical classes of the *C. sativa* and *Castanea mollissima* complex

	Cs	***Cc***	***F*$_1$(*CcxCs*)**	***Cm***
Cs	*Cs*			
Cc	F_1(*CcxCs*)	*Cc*		
F_1(*CcxCs*)	*BC*(*Cs*)	*BC*(*Cc*)	F_2(*CcxCs*)	
Cm	F_1(*CmxCs*)			*Cm*

Cs: Castanea sativa; Cc: Castanea crenata; Cm: Castanea mollissima; F_1(*CcxCs*): first generation hybrids between *C. sativa* and *C. crenata;* F_1(*CmxCs*): first generation hybrids between *C. sativa* and *C. mollissima;* F_2(*CcxCs*): second generation hybrids between *C. sativa* and *C. crenata; BC*(*Cs*) and *BC*(*Cc*) are the backcrosses between the F_1(*CcxCs*) on *Cs* and *Cc,* respectively.

Genotype inspection (*GI*) of the diagnostic loci for each genotyped individual was performed to assign them to genotypic classes following the descriptions of Nasson and Ellstrand (1993). The genotypic classes typical of each parental species have all of the alleles that are diagnostic of the species and no allele of the other species; first generation hybrids have genotypes that are heterozygous for all of the diagnostic *loci*; the typical second generation hybrids have at least one locus that is homozygous for alleles unique to one parental species and at least one locus homozygous for alleles unique to the other parental species; genotypes typical of backcrosses have at least one diagnostic *locus* that is homozygous for the parental species to which the individual is backcrossed and at least one other diagnostic *locus* that is heterozygous for diagnostic alleles of the two species involved. The genotypic classes typical of parental, F_1 and *BC* are also present in individuals of F_2 genealogic classes and parental and F_1 genotypic classes are present in individuals belonging to *BC*.

The percentage of ancestry of each individual in each species (*C. sativa*, *C. crenata* and *C. mollissima*) was estimated with *STRUCTURE* software version 2.2 (Pritchard *et al.,* 2000; Falush *et al.,* 2007) for K = 3. An admixture model with a burning period of 40,000, followed by 40,000 iterations was used. The number of individuals that were entirely morphologically identified as *Cs*, *Cc* and *Cm* were 45, 37 and 27, respectively (Table 1), and these were used as reference samples (learning samples) with the option in which allele frequencies are updated only for individuals with *Popflag* = 1, and allele frequencies between the different involved species were considered uncorrelated. Ten independent runs were carried out and were analysed using *CLUMPP* version 1.1.1 software (Jakobsson and Rosenberg, 2007), which estimates the mean ancestry across independent runs of each individual in each K = 3 species. A histogram displaying the ancestry of each individual in each species was constructed with *DISTRUCT* software (Rosenberg, 2004). The number of loci analyzed was 13, including one locus (*Pgm-2*) with null alleles. The value of the ancestry of each individual in one of the hybridising species (*q*) was considered a hybrid index. The threshold values of the reference samples of pure parental species, as identified morphologically, estimated with *STRUCTURE* were used to assign individuals to *Cs*, *Cc*, or *Cm* if their ancestry in the species was equal or greater than the ancestry of reference samples. Individuals with ances-

try values lower than those of the reference samples were identified as hybrids between the species for which they showed some ancestry, and these individuals were identified, for example, as *hybrid* (*Cc, Cs*).

Estimation of each individual's probability of belonging to each genealogical class was performed with *NEWHYBRIDS* Version 1.1. beta (Anderson, 2002). A burnin period of 40,000, followed by 40,000 iterations was used. This analysis was performed to identify genealogical classes between *C. sativa* and *C. crenata* and, as a consequence reference samples of *C. mollissima* were excluded. Prior information from pure *C. sativa* and *C. crenata* was used with the *z* option. A locus with a null allele (*Pgm-2*) was removed from the analysis. The hybrid indices that were obtained from *NEWHYBRIDS* are the probabilities in each genealogic class [*Cs*, *Cc*, F_1(*CcxCs*), F_2(*CcxCs*), *BC*(*Cs*), *BC*(*Cc*)]. An individual was assigned to a genealogic class if the probability in this genealogic class was ≥ 0.50, and it was designated as an *undetermined hybrid* if its probabilities of belonging to different genealogical classes were under 0.50.

The analyses using *STRUCTURE* and *NEWHYBRIDS* were performed separately for the samples genotyped for all loci (341 individuals) and for the hybrids from *EFAC*, which were genotyped with a reduced number of loci (112 individuals).

For the final assignation of individuals to genealogical classes, the following criteria were considered:

— Individuals were assigned to *C. sativa* or to *C. crenata* if they were assigned to these species by the three methods (*GI, STRUCTURE* and *NEWHYBRIDS*) and their ancestry and probability of belonging to *Cs* and *Cc* (estimated with *STRUCTURE* and *NEWHYBRIDS*, respectively) were ≥ than the threshold values of the reference samples. For *Cm*, only *GI* and *STRUCTURE* were used.

— An individual was assigned to *GC* F_1(*CcxCs*) if the following conditions were fulfilled: its genotype class was typical of F_1(*CcxCs*) by *GI*; it was classified as *hybrid* (*Cc, Cs*) by STRUCTURE; and it was classified as F_1(*CcxCs*) by *NEWHYBRIDS*.

— Individuals assigned to *GC* F_1(*CcxCs*) by *GI* that were classified as F_2(*CcxCs*) or undetermined with *NEWHYBRIDS* were classified as F_2(*CcxCs*)-*BC*(*Cs*)-*BC*(*Cc*).

— The genotypic class typical of F_2 plants was present only in individuals of the genealogical class F_2. As a consequence, individuals classified as F_2 by genotype inspection belonged to the genealogical class F_2.

— Individuals classified as *BC* by the inspection method could be F_2(*CcxCs*) or *BC* and were assigned to the group F_2(*CcxCs*)-*BC*(*Cs*) or F_2(*CcxCs*)-*BC*(*Cc*).

To determine the heterozygosity of individuals classified into the different genealogical classes, the observed heterozygosity (*Ho*) of each individual was estimated with *GENALEX*. The presence of perfect or collapsed glandular trichomes was determined in each individual of the clone collection, and its frequency was computed for the different genealogical classes that were identified.

Results

Species diversity and differentiation

Banding patterns were obtained for all enzyme systems for a total of 21 *loci*: two *loci* for aconitase (*Aco-1*, *Aco-2*), phosphoglucoisomerase (*Pgi-1*, *Pgi-2*), phosphoglucomutase (*Pgm-1*, *Pgm-2*), isocitrate dehydrogenase (*Idh-1*, *Idh-2*), phosphogluconate dehydrogenase (*Pgd-1*, *Pgd-2*) and fructose biphosphate (*Fdp-1*, *Fdp-2*); three *loci* in malate dehydrogenase (*Mdh-1*, *Mdh-2*, *Mdh-3*) and *UTP*-glucose-1-phosphate-uridyltransferase (*Ugp-1*, *Ugp-2* and *Ugp-3*); and one *locus* in shikimate dehydrogenase (*Sdh*), diaphorase (*Dia-1*) and peroxidase (*Prx-3*). Most enzymes were obtained from buds and leaves, with the exception of *Idh-1*, *Ugp-2* and *Ugp-3*, which were extracted only from buds, and *Aco-1* and *Aco-2*, which were extracted only from leaves. *Aco-2* and *Ugp-2* were not used in further analyses because of a scarcity of data being produced for these loci. The *loci* that were monomorphic for the studied group of species were *Pgi-1*, *Pgm-1*, *Mdh-1*, *Pgd-1*, *Fdp-1*, *Fdp-2*.

The genetic diversity of the studied populations of introduced Japanese and Chinese chestnuts was lower than the diversity of the sweet chestnut population. The ranking of species by their expected heterozygosity was: sweet chestnut ($He = 0.28 \pm 0.08$) > Japanese chestnut ($He = 0.17 \pm 0.05$) > Chinese chestnut ($He = 0.09 \pm 0.04$). The ranking by polymorphism was: sweet chestnut ($P = 69.23\%$) > Japanese chestnut ($P = 61.54\%$) > Chinese chestnut ($P = 46.15\%$). The ranking of F_{st} values between pairs of species was: sweet-Chinese ($F_{st} = 0.43$) > Japanese-Chinese ($F_{st} = 0.36$) > sweet-Japanese ($F_{st} = 0.28$).

The allelic frequencies estimated for the reference samples (Table 3) allowed us to distinguish different

Table 3. The ascertained allele frequencies within reference samples of European chestnut (*Cs*), Japanese chestnut (*Cc*) and Chinese chestnut (*Cm*) for eleven loci are useful as diagnostic loci (fixed in one species) or as diagnostic alleles (exclusive of one species, but not fixed)

Locus[2]	Allele	Species[1]			Exclusive alleles	Diagnostic loci
		Cs	*Cc*	*Cm*		
Aco-1	102			0.98	*Cm*	Differentiates *Cs* and *Cc* from *Cm,*
	100	1.00	1.00	0.02		but not entirely
Pgi-2	108	0.51			*Cs*	Differentiated *Cc* from *Cs* and *Cm*,
	105	0.21	0.05	0.64		but not entirely
	100	0.28		0.04		
	95		0.95		*Cc*	
	90			0.32	*Cm*	
Pgm-2	102		0.06		*Cc*	Null allele in *Cs* differentiates *Cs* from
	100		0.88	0.94	*Cc, Cm*	*Cc* and *Cm*
	98		0.03		*Cc*	
	90			0.06	*Cm*	
	Null	1.00	0.03			
Mdh-2	105	0.24			*Cs*	
	100	0.76	1.00	1.00		
Mdh-3	100	0.90	1.00	1.00		
	95	0.10			*Cs*	
Sdh-1	100	0.46			*Cs*	Differentiates *Cs* from *Cc* and *Cm*
	97	0.19			*Cs*	
	95	0.35			*Cs*	
	90		1.00	1.00	*Cc, Cm*	
Idh-1	105	0.37	0.06	0.00		
	100	0.63	0.94	1.00		
Idh-2	105		0.81	0.27	*Cc, Cm*	
	100	1.00	0.19	0.73		
Pgd-2	105	1.00	0.84	0.06		
	100		0.16	0.94	*Cc, Cm*	
Dia-1	110	0.39	0.00	0.00	*Cs*	
	100	0.61	1.00	1.00		
Ugp-1	105	0.12			*Cs*	
	100	0.88	0.69	1.00		
	95		0.31		*Cc*	
Ugp-3	100	1.00	0.87	0.04		
	95		0.13	0.96	*Cc, Cm*	
Prx-3	106	0.15			*Cs*	
	104	0.04	0.36			
	102	0.28	0.64	1.00		
	100	0.33			*Cs*	
	95	0.20			*Cs*	

[1] *Cs: Castanea sativa; Cc: Castanea crenata; Cm: Castanea mollissima.* [2] *Aco-1* (aconitase); *Pgi-2* (phosphoglucoisomerase); *Pgm-2* (phosphoglucomutase); *Mdh-2* and *Mdh-3* (malate dehydrogenase); *Sdh-1* (shikimate dehydrogenase); *Idh-1, Idh-2* (isocitrate dehydrogenase); *Pgd-2* (phosphogluconate dehydrogenase); *Dia-1* (diaphorase); *Ugp-1, Ugp-2* (UTP-glucose-1-phosphate-uridyltransferase); *Prx-3* (peroxidase).

types of loci with regard to their potential use for species identification. Among 37 alleles, 17 were exclusive to one species: eleven alleles were exclusive to *C. sativa* (alleles *Pgi-2*^{108}; *Mdh-2*^{105}; *Mdh-3*^{95}; *Sdh-1*^{100}, *Sdh-1*^{97}, *Sdh-1*^{95}; *Dia-1*^{110};*Ugp-1*^{105}; *Prx-3*^{106}, *Prx-3*^{100}, *Prx-3*^{95}); four were exclusive to *C. crenata* (*Pgi-2*^{95}; *Pgm-2*^{102}, *Pgm-2*^{98}, *Ugp-1*^{95}); two alleles were exclusive to *C. mollissima* (*Pgi-2*^{90}, *Pgm-2*^{90}); and four additional alleles were exclusive to both Asiatic species (alleles *Pgm-2*^{100}, *Sdh-1*^{90}, *Idh-2*^{105} and *Pgd-2*^{100}). The unique diagnostic *locus* found was *Sdh*, though three additional *loci* (*Aco-1*, *Pgm-2* and *Pgi-2*) were almost fixed for different species.

Assignation of individuals to genotypic classes with the GI

The *loci* used for the classification by *GI* were *Aco-1*, *Pgi-2*, *Pgm-2* and *Sdh*. The hybrids between *C. sativa* and *C. crenata* always presented *Aco-1*^{100} in homozygosis, and *Sdh* and *Pgi-2* were used as diagnostic *loci* to assign genotypes to genotypic classes. Individuals with the allele *Aco-2*^{102} in homocygosis were assigned to *C. mollissima*. The locus *Pgm-2,* for which there was a null allele in *C. sativa,* was used to confirm the presence of Asiatic germplasm, as the allele *Pgm-2*^{100} must be present in most individuals of *C. crenata* and *C. mollissima* and in most F_1 plants. The classification produced the following groupings: *C. sativa* with 69 individuals; *C. crenata* with 58 individuals; *C. mollissima* with 26 individuals; F_1(*CcxCm*) with three individuals; F_1(*CcxCs*) with 71 individuals; F_2(*CcxCs*) with 9 individuals; *BC*(*Cs*) with 79 individuals; *BC*(*Cc*) with 21 individuals; and 5 individuals with no assignation.

Identification of the parental species involved in crosses with STRUCTURE

The output of *STRUCTURE* for 10 runs was repetitive, as indicated by the results of *CLUMPP*, which gave a very high average pair-wise similarity value (*H'* = 0.99). The average assignments of individuals determined by *CLUMPP* indicated the ancestry of each individual in each of the three species, as shown in Figure 2. Each individual is represented by a vertical bar in which the percentages of its ancestry in *C. sativa*, *C. crenata* and *C. mollissima* are represented in yellow, green and orange, respectively. As is evident from Figure 2, most individuals in the Lourizán Clone Collection exhibited shared genetic ancestry between C. *sativa* and *C. crenata*. There were only three individuals that shared genetic ancestry between *C. mollissima* and *C. sativa*, and several individuals showed ancestry only in *C. crenata* or in *C. sativa*. Several F_1 (*Cc* × *Cs*) individuals of known genealogy, in the histogram, demonstrated proportions of ancestry from the two parental species very close to 0.5:0.5. The data presented in Figure 2 indicate that most individuals in the Clone Collection, have proportions of *C. sativa* and *C. crenata* ancestry that are far from the proportion

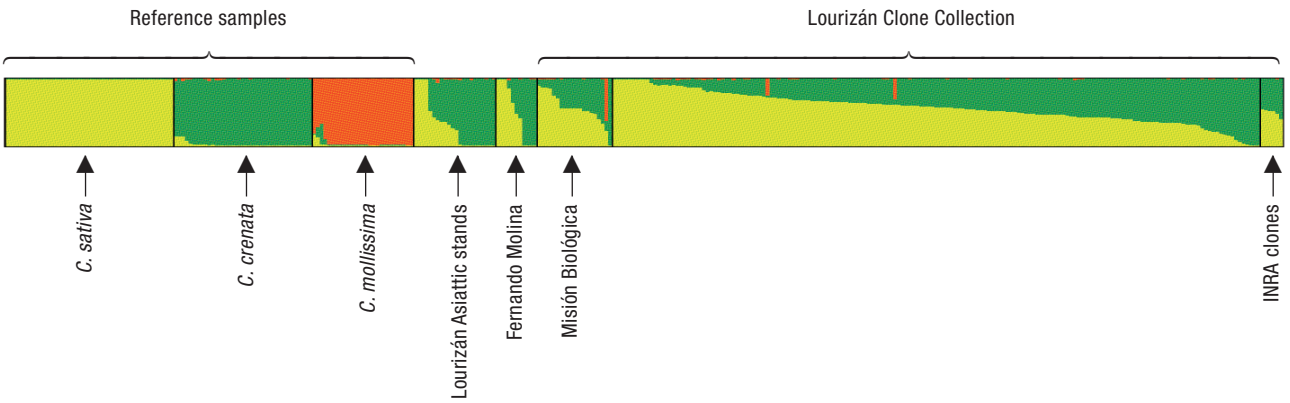

Figure 2. Ancestry of 341 individuals in $K = 3$ populations estimated with *STRUCTURE*. Each individual is represented by a vertical bar in which the percentage of ancestry of each individual in each original population is shown by different colours: sweet chestnut (*C. sativa*) in yellow, Japanese chestnut (*C. crenata*) in green, or Chinese chestnut (*C. mollissima*) in orange. Individuals within each population were arranged by their decreasing ancestry in *C. sativa*. Most individuals included the Lourizán Clone Collection were classified as interspecific hybrids between *C. crenata* and *C. sativa* but a few were classified as pure *C. sativa* or as pure *C. crenata.*

0.5:0.5 that is expected in F_1 crosses, and consequently, these individuals must be F_2, F_3 or backcrosses to *C. crenata* or to *C. sativa*. The threshold values of ancestry of pure *C. sativa*, *C. crenata* and *C. mollissima* that were determined with *STRUCTURE* were 0.98, 0.91 and 0.82, respectively. These threshold values were used as reference values for the assignation of individuals to *Cs*, *Cc*, *Cm* and to hybrids.

The classification produced the following groups: *C. sativa* with 62 individuals, *C. crenata* with 55 individuals, *C. mollissima* with 25 individuals, *hybrids* (*Cc, Cs*) with 194 individuals, *hybrids* (*Cm, Cs*) with 2 individuals, *hybrids* (*Cc, Cm*) with 1 individual and *hybrids* (*Cc, Cm, Cs*) with 2 individuals.

The assignation of individuals to genealogical classes with NEWHYBRIDS

Reference samples of *C. mollissima* were removed from further analysis with *NEWHYBRIDS* because this software is defined for the analysis of hybrids between two species. The assignation of genotyped individuals with *NEWHYBRIDS* resulted in classification of individuals into four groups: *C. sativa* with 89 individuals; *C. crenata* with 61 individuals; F_1(*CcxCs*) with 96 individuals; F_2(*CcxCs*) with 38 individuals; and 31 individuals were not assigned to any group because their probability in each genealogic class was lower than 0.50; no individual was assigned as having resulted from backcrosses to *C. sativa* or to *C. crenata*. The lowest probabilities for reference samples of being *Cs* and *Cc* determined with *NEWHYBRIDS* were 0.88 and 0.70, respectively.

Assignation to genealogical classes using the three methods

All 356 individuals were assigned to one of the following ten groups: *Cs*, *Cc*, *Cm*, F_1(*CcxCs*), F_2(*CcxCs*), F_2(*CcxCs*)-*BC*(*Cs*), F_2(*CcxCs*)-*BC*(*Cc*), F_2(*CcxCs*)-*BC*(*Cs*)-*BC*(*Cc*), *hybrid* (*Cc, Cm*) and *hybrid* (*Cs, Cm*). All 45 reference samples that were classified as *Cs* based on morphology were also classified as *C. sativa* using all three methods; among 37 reference samples of *C. crenata*, 34 were classified as *Cc* by the three methods, while three individuals were excluded as *Cc* only with *NEWHYBRIDS* (two were identified as F_2(*CcxCs*), and one was not assigned; among 27 reference samples of *Cm*, 25 were classified as *Cm*, and two (*M-14* and *MB-1*) were considered not to be pure *Cm* based on *STRUCTURE*.

The classification of individuals from the different studied populations is summarized in Table 4.

Table 4. Assignation of samples of studied populations to different genealogic classes or groups of genealogical classes (example: F_2(*CcxCs*)-*BC*(*Cs*) includes individuals that could belong to some of these genealogical classes) estimated by genotype inspection of diagnostic loci combined with the Bayesian procedures implemented in *NEWHYBRIDS* and *STRUCTURE*

Population	No. trees	*Cs*	*Cc*	*Cm*	F_1(*CcxCs*)	F_2(*CcxCs*)	F_2(*CcxCs*) *BC*(*Cs*)	F_2(*CcxCs*) *BC*(*Cc*)	F_2(*CcxCs*) *BC*(*Cs*) *BC*(*Cc*)	*H*(*Cc,Cm*)	*H*(*Cm,Cs*)
Wild Galician	36	36									
Romanian clone	9	9									
Granxa da Serra	15	3	11					1			
Prado das Troitas	21		15		4			2			
As Minas	17		13		4						
Agrovello	21	1	5	15							
Fernando Molina	12	3	4		2		1		1	1	
Misión Biológica	27		1	7	10	1	6		1		1
INRA arboretum	4			4							
MB + EFAC clone	173	9	6		38	9	81	23	5		2
INRA clone	6				4				2		
EFAC clone	15				12		3				
Total	356	61	55	26	74	10	91	26	9	1	3

Cs: Castanea sativa; Cc: C. crenata; Cm: C. mollissima; F_1(*CcxCs*): first generation hybrid between *C. sativa* and *C. crenata;* F_2(*CcxCs*): second generation hybrid between *C. crenata* and *C. sativa;* *BC*(*Cs*): backcross of F_1(*CcxCs*) on *C. sativa;* *BC*(*Cc*): backcross of F_1(*CcxCs*) on *C. crenata;* *H*(*Cc,Cm*) and *H*(*Cm,Cs*): hybrid between *Cc* and *Cm* or between *Cm* and *Cs,* respectively.

The Lourizán Asiatic chestnut stands contain mainly *Cc* and the Agrovello stand contains mainly *Cm*, although in both cases there are several *Cs* individuals and a few F_1(*CcxCs*) and F_2(*CcxCs*)-*BC*(*Cc*). In the Fernando Molina file most individuals were classified according to expectations, however two individuals (*M-7* and *M-11*) differ of genotypes expected (Table 5a). In the *MB* line 18 *hybrid* (*Cc, Cs*) individuals were identified (Table 5.b), and although 10 of these were classified as F_1(*CcxCs*), the presence of the null allele in *Pgm-2* and the absence of collapsed glandular trichomes on the abaxial surfaces of leaves indicated that the *MB* hybrids are probably F_2(*CcxCs*) or *BC*. Most of the clones of the Lourizán clone collection, the *EFAC+MB* clones, were *hybrid* (*Cc, Cs*) (171 clones), and only two clones were *hybrid* (*Cm, Cs*). Among these 171 clones, 38 were classified as F_1(*CcxCs*), and all the other clones were classified as F_2(*CcxCs*) or *BC*, most of which were to *Cs* (Table 4 and Table 5c). Within this subset, there are 18 clones approved as basic material (Table 5c). Among the *INRA* clones, four were classified as F_1(*CcxCs*), and two were assigned to the group F_2(*CcxCs*)-*BC*(*Cs*)-*BC*(*Cc*) (Table 4 and Table 5c). Among the 15 *EFAC* clones, 12 were classified as F_1(*CcxCs*), as expected, and three were classified as F_2(*CcxCs*)-*BC*(*Cs*) (Table 4 and Table 5c).

Heterozygosis

The observed heterozygosity was higher for most hybrids than for pure species, as expected, and this is summarised in Figure 3. The highest heterozygosity

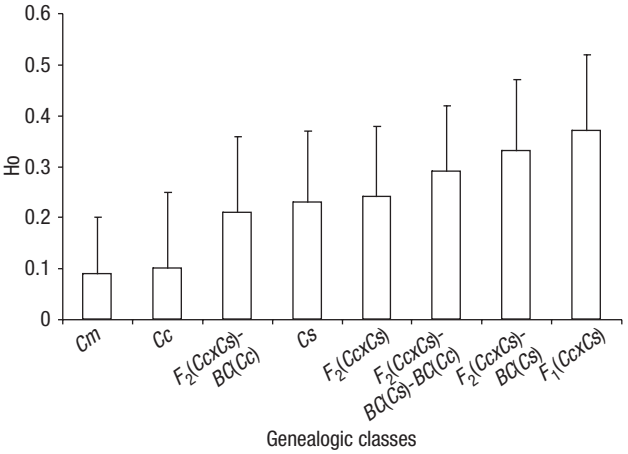

Figure 3. Mean observed heterozygosis and its standard deviation calculated for individuals classified into the different genealogic classes or groups of genealogic classes between *C. sativa* and *C. crenata. Cs: Castanea sativa; Cc: C. crenata; Cm: C. mollissima;* F_1(*CcxCs*): first generation hybrid between *C. sativa* and *C. crenata;* F_2(*CcxCs*): second generation hybrid between *C. crenata* and *C. sativa; BC*(*Cs*): backcross of F_1(*CcxCs*) on *C. sativa; BC*(*Cc*): backcross of F_1(*CcxCs*) on *C. crenata.*

Table 5. Classification using genotype inspection, *STRUCTURE*, *NEWHYBRIDS*, a final classification and heterozygosis of individuals: a) Fernando Molina line in Lourizán; b) Misión Biológica line; c) clones approved as basic material in the categories Tested or Qualified of the Lourizán Clone Collection, including *MB + EFAC* clones, *EFAC* clones and two *INRA* French clones

a) Fernando Molina Line

Tree	Expected genealogic class	Genotype inspection Classification 1	*STRUCTURE* (% ancestry in each species)				*NEWHYBRIDS* (probability in each genealogical class)							Final classification	Ho
			Cs	*Cc*	*Cm*	Classification 2	*Cs*	*Cc*	F_1	F_2	*BC*(*Cc*)	*BC*(*Cs*)	Classification 3		
M-1	*Cs*	*Cs*	0.99	0.01	0.00	*Cs*	0.99	0.00	0.00	0.00	0.00	0.01	*Cs*	*Cs*	0.385
M-2	F_1(*CcxCs*)	F_1(*CcxCs*)	0.64	0.36	0.00	*Hybrid* (*Cs, Cc*)	0.00	0.00	0.71	0.22	0.00	0.07	F_1(*CcxCs*)	F_1(*CcxCs*)	0.385
M-3	*Cc*	*Cc*	0.01	0.99	0.00	*Cc*	0.00	0.99	0.00	0.00	0.00	0.00	*Cc*	*Cc*	0.000
M-4	*Cs*	*Cs*	0.98	0.01	0.00	*Cs*	0.95	0.00	0.00	0.02	0.00	0.03	*Cs*	*Cs*	0.231
M-5	F_1(*CcxCs*)	F_1(*CcxCs*)	0.41	0.57	0.02	*Hybrid* (*Cs, Cc*)	0.00	0.00	0.92	0.07	0.00	0.01	F_1(*CcxCs*)	F_1(*CcxCs*)	0.462
M-6	*Cc*	*Cc*	0.01	0.99	0.01	*Cc*	0.00	1.00	0.00	0.00	0.00	0.00	*Cc*	*Cc*	0.154
M-7	*Cs*	*Cs*	0.84	0.12	0.03	*Hybrid* (*Cs, Cc*)	0.83	0.00	0.01	0.06	0.00	0.10	*Cs*	F_2(*CcxCs*)-*BC*(*Cs*)	0.231
M-9	*Cc*	*Cc*	0.01	0.97	0.02	*Cc*	0.00	1.00	0.00	0.00	0.00	0.00	*Cc*	*Cc*	0.000
M-10	*Cs*	*Cs*	0.98	0.01	0.00	*Cs*	0.95	0.00	0.00	0.01	0.00	0.03	*Cs*	*Cs*	0.308
M-11	F_1(*CcxCs*)	*Indeterminate*	0.48	0.51	0.00	*Hybrid* (*Cs, Cc*)	0.03	0.00	0.25	0.57	0.00	0.15	F_2(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.154
M-14	*Cm*	*Cm*	0.03	0.30	0.66	*Hybrid* (*Cc, Cm*)	0.00	0.62	0.09	0.25	0.04	0.00	*Cc*	*Hybrid* (*Cc, Cm*)	0.308
M-15	*Cc*	*Cc*	0.01	0.98	0.00	*Cc*	0.00	0.97	0.00	0.02	0.01	0.00	*Cc*	*Cc*	0.154

b) Misión Biológica line

Tree	Genotype inspection Classification 1	*STRUCTURE* (% ancestry in each species)				*NEWHYBRIDS* (probability in each genealogical class)							Final classification	Ho
		Cs	*Cc*	*Cm*	Classification 2	*Cs*	*Cc*	F_1	F_2	*BC*(*Cc*)	*BC*(*Cs*)	Classification 3		
MB-1	*Cm*	0.14	0.14	0.73	*Hybrid* (*Cs*,*Cc*,*Cm*)	—	—	—	—	—	—	*Cm*	*ver*	0.077
MB-2	*Cc*	0.01	0.98	0.00	*Cc*	0.00	0.98	0.00	0.02	0.01	0.00	*Cc*	*Cc*	0.000
MB-3	*BC*(*Cs*)	0.71	0.29	0.00	*Hybrid* (*Cs*,*Cc*)	0.28	0.00	0.17	0.34	0.00	0.20	*Indetermined*	F_2(*CcxCs*)-*BC*(*Cs*)	0.077
MB-4	F_1(*CcxCs*)	0.33	0.65	0.02	*Hybrid* (*Cs*,*Cc*)	0.00	0.02	0.30	0.60	0.07	0.00	F_2(*CcxCs*)	F_2(*CcxCs*)	0.231
MB-5	*Cm*	0.01	0.01	0.98	*Cm*	—	—	—	—	—	—	—	*Cm*	0.077
MB-6	*Cm*	0.01	0.01	0.98	*Cm*	—	—	—	—	—	—	—	*Cm*	0.077
MB-7	F_2(*CcxCs*)	0.56	0.43	0.01	*Hybrid* (*Cs*,*Cc*)	0.02	0.01	0.07	0.84	0.01	0.05	F_2(*CcxCs*)	F_2(*CcxCs*)	0.154
MB-8	F_1(*CcxCs*)	0.49	0.51	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.00	0.86	0.10	0.00	0.03	F_1(*CcxCs*)	F_1(*CcxCs*)	0.308
MB-9	*Cs*	0.86	0.14	0.01	*Hybrid* (*Cs*,*Cc*)	0.67	0.00	0.03	0.18	0.00	0.13	*Cs*	F_2(*CcxCs*)	0.154
MB-10	F_1(*CcxCs*)	0.40	0.59	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.00	0.86	0.10	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)	0.231
MB-11	BC(*Cs*)	0.88	0.11	0.02	*Hybrid* (*Cs*,*Cc*)	0.75	0.00	0.02	0.10	0.00	0.13	*Cs*	F_2(*CcxCs*)	0.231
MB-12	F_1(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.00	0.86	0.10	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-13	F_1(*CcxCs*)	0.48	0.52	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.00	0.86	0.10	0.00	0.03	F_1(*CcxCs*)	F_1(*CcxCs*)	0.308
MB-14	BC(*Cs*)	0.75	0.24	0.01	*Hybrid* (*Cs*,*Cc*)	0.24	0.00	0.23	0.34	0.00	0.19	*Indetermined*	F_2(*CcxCs*)-*BC*(*Cs*)	0.231
MB-15	F_1(*CcxCs*)	0.52	0.48	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.00	0.60	0.36	0.02	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-16	*Cm*	0.17	0.01	0.82	*Cm*	—	—	—	—	—	—	—	*Cm*	0.000
MB-17	F_1(*CcxCs*)	0.53	0.47	0.01	*Hybrid* (*Cs*,*Cc*)	0.03	0.00	0.57	0.28	0.00	0.11	F_1(*CcxCs*)	F_1(*CcxCs*)	0.154
MB-18	F_2(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.01	0.84	0.12	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-19	F_2(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.01	0.84	0.12	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-20	F_2(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.01	0.84	0.12	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-21	*Cs*	0.88	0.12	0.01	*Hybrid* (*Cs*,*Cc*)	0.79	0.00	0.00	0.09	0.00	0.11	*Cs*	F_2(*CcxCs*)-*BC*(*Cs*)	0.231
MB-22	*Cs*	0.89	0.10	0.01	*Hybrid* (*Cs*,*Cc*)	0.88	0.00	0.00	0.04	0.00	0.08	*Cs*	F_2(*CcxCs*)-*BC*(*Cs*)	0.154
MB-23	F_1(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs*,*Cc*)	0.00	0.01	0.84	0.12	0.01	0.02	F_1(*CcxCs*)	F_1(*CcxCs*)*	0.231
MB-s1	*Cm*	0.01	0.02	0.98	*Cm*	—	—	—	—	—	—	—	*Cm*	0.154
MB-s2	*Cm*	0.01	0.01	0.98	*Cm*	—	—	—	—	—	—	—	*Cm*	0.077
MB-s3	*Cm*	0.23	0.15	0.63	*Hybrid* (*Cs*,*Cc*,*Cm*)	0.00	0.34	0.27	0.34	0.03	0.02	*Indetermined*	*Cm*	0.154
MB-s4	*Hybrid*(*Cm*, *Cs*)	0.01	0.02	0.97	*Hybrid* (*Cs*,*Cc*)	—	—	—	—	—	—	—	*Hybrid* (*Cm*, *Cs*)	0.154

was found in F_1(*CcxCs*), with values between 0.15 and 0.69, followed by the group F_2(*CcxCs*)-*BC*(*Cs*), with values between 0.08 and 0.54; the lowest values were observed in *Cm*, which exhibited values between 0 and 0.23, and in *Cc*, with values between 0 and 0.31.

Perfect and collapsed glandular trichomes in hybrids

Some individuals belonging to the Clone Collection that were classified as *C. sativa* displayed the collapsed glandular trichomes of hybrids (3 out of 8). This also occurred in individuals with multilocus genotypes of pure *C. crenata*: two out of four individuals displayed the perfect type of glandular trichome, one the collapsed type, and one did not present any glandular trichomes. Among interspecific hybrids of different categories, the presence of glandular trichomes was observed in 76.3% of individuals classified as F_2(*CcxCs*)-*BC*(*Cc*), in 51.7% of individuals classified as F_1(*CcxCs*) and in 23.9% of *F2*(*CcxCs*)-*BC*(*Cs*) plants. In all cases, most of these glandular trichomes were of the collapsed type.

Discussion

Diversity of introduced Asiatic germplasm

The values for genetic distances obtained in this study confirm the higher level of genetic relatedness between the two Asiatic species compared with the European chestnut, as expected. The closer genetic proximity of *C. sativa* to *C. crenata*, as opposed to *C. mollissima*, contradicts results that were obtained using *cpDNA* (Lang *et al*., 2006). The low genetic variability of the Asiatic populations that were studied may be due to low variability in the seed lots that were introduced into Spain. Most introductions of Japanese chestnuts into Europe were of the variety 'Tamba'.

c) Lourizán Clone Collection

Clone	Number in Clone Collection	Genotype inspection Classification 1	*STRUCTURE* (% ancestry in each species)				*NEWHYBRIDS* (probability in each genealogical class)							Final classification	Ho	Basic material
			Cs	*Cc*	*Cm*	Classification 2	*Cs*	*Cc*	F_1	F_2	*BC*(*Cc*)	*BC*(*Cs*)	Classification 3			
MB+EFAC																
514	1	*BC*(*Cs*)	0.65	0.35	0.00	*Hybrid* (*Cs,Cc*)	0.03	0.00	0.01	0.87	0.00	0.09	F_2(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.231	Tested
513	5	*BC*(*Cs*)	0.53	0.46	0.00	*Hybrid* (*Cs,Cc*)	0.02	0.00	0.60	0.28	0.00	0.10	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.231	Tested
374	27	F_1(*CcxCs*)	0.66	0.34	0.00	*Hybrid* (*Cs,Cc*)	0.11	0.00	0.18	0.47	0.00	0.24	*Indeterminate*	F_2(*CcxCs*)-*BC*(*Cs*)	0.231	Qualified
111-1	33	*BC*(*Cs*)	0.46	0.54	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.01	0.54	0.40	0.05	0.01	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.231	Tested
X	35	*BC*(*Cc*)	0.67	0.33	0.00	*Hybrid* (*Cs, Cc*)	0.00	0.00	0.09	0.89	0.01	0.02	F_2(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cc*)	0.308	Tested
16	39	*BC*(*Cs*)	0.90	0.09	0.01	*Hybrid* (*Cs, Cc*)	0.85	0.00	0.01	0.03	0.00	0.11	*Cs*	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
942	44	*BC*(*Cs*)	0.75	0.24	0.01	*Hybrid* (*Cs, Cc*)	0.29	0.00	0.14	0.31	0.00	0.25	*Indeterminate*	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
2003	114	*BC*(*Cs*)	0.60	0.39	0.00	*Hybrid* (*Cs, Cc*)	0.00	0.00	0.88	0.09	0.00	0.03	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
2522	117	F_2(*CcxCs*)	0.79	0.21	0.00	*Hybrid* (*Cs, Cc*)	0.06	0.00	0.01	0.87	0.00	0.06	F_2(*CcxCs*)	F_2(*CcxCs*)	0.154	Tested
U-125	137	F_1(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs, Cc*)	0.00	0.00	0.90	0.06	0.00	0.04	F_1(*CcxCs*)	F_1(*CcxCs*)	0.462	Qualified
FB-1-S-P	141	*BC*(*Cs*)	0.61	0.38	0.01	*Hybrid* (*Cs, Cc*)	0.16	0.00	0.24	0.40	0.00	0.20	*Indeterminate*	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
431	147	*Cs*	0.99	0.01	0.00	*Cs*	0.92	0.00	0.00	0.03	0.00	0.04	*Cs*	*Cs*	0.077	Tested
U-90025	149	F_1(*CcxCs*)	0.56	0.44	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.90	0.06	0.00	0.04	F_1(*CcxCs*)	F_1(*CcxCs*)	0.462	Tested
H-S	151	*BC*(*Cs*)	0.65	0.34	0.01	*Hybrid* (*Cs,Cc*)	0.32	0.00	0.01	0.48	0.00	0.18	*Indeterminate*	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
130	153	*Cs*	0.99	0.01	0.00	*Cs*	0.99	0.00	0.00	0.00	0.00	0.01	*Cs*	*Cs*	0.231	Tested
U-7521	162	*Hybrid* (*Cm,Cs*)	0.61	0.08	0.31	*Hybrid* (*Cs,Cm*)	0.32	0.00	0.41	0.16	0.00	0.11	*Indeterminate*	*Hybrid* (*CmxCs*)	0.385	Tested
420	164	*BC*(*Cs*)	0.61	0.39	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.86	0.11	0.00	0.03	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.385	Tested
U-19	167	F_1(*CcxCs*)	0.39	0.60	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.30	0.65	0.05	0.00	F_2(*CcxCs*)	F_2(*CcxCs*)	0.615	Tested
EFAC																
U-1482	179	F_1(*CcxCs*)	0.50	0.50	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.02	0.77	0.13	0.02	0.07	F_1(*CcxCs*)	F_1(*CcxCs*)	0.33	Qualified
U-1483	180	F_1(*CcxCs*)	0.61	0.39	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.97	0.01	0.02	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-324	181	F_1(*CcxCs*)	0.54	0.45	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.98	0.01	0.01	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.67	Qualified
U-374	182	F_1(*CcxCs*)	0.42	0.57	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.03	0.78	0.11	0.01	0.07	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-392	183	F_1(*CcxCs*)	0.54	0.45	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.98	0.01	0.01	0.00	F_1(*CcxCs*)	$F_1$1(*CcxCs*)	0.67	Qualified
U-3	184	F_1(*CcxCs*)	0.61	0.38	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.97	0.01	0.02	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-55	187	F_1(*CcxCs*)	0.54	0.45	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.98	0.01	0.01	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.67	Qualified
U-592	188	*BC*(*Cs*)	0.68	0.22	0.09	*Hybrid* (*Cs,Cc*)	0.04	0.00	0.65	0.06	0.24	0.00	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.50	Qualified
U-70005	189	F_1(*CcxCs*)	0.61	0.38	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.97	0.00	0.02	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-70007	190	F_1(*CcxCs*)	0.61	0.38	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.97	0.01	0.02	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-760	192	F_1(*CcxCs*)	0.50	0.37	0.14	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.98	0.01	0.01	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.67	Qualified
U-7810	193	*BC*(*Cs*)	0.74	0.26	0.00	*Hybrid* (*Cs,Cc*)	0.04	0.00	0.65	0.07	0.24	0.00	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.50	Qualified
U-88	195	*BC*(*Cs*)	0.69	0.31	0.00	*Hybrid* (*Cs,Cc*)	0.06	0.00	0.53	0.11	0.30	0.00	F_1(*CcxCs*)	F_2(*CcxCs*)-*BC*(*Cs*)	0.67	Qualified
U-89	196	F_1(*CcxCs*)	0.55	0.45	0.00	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.98	0.01	0.02	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.50	Qualified
U-90044	197	F_1(*CcxCs*)	0.73	0.26	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.87	0.03	0.11	0.00	F_1(*CcxCs*)	F_1(*CcxCs*)	0.33	Qualified
INRA																
CA-15	—	F_1(*CcxCs*)	0.49	0.51	0.01	*Hybrid* (*Cs,Cc*)	0.00	0.00	0.94	0.04	0.00	0.01	F_1(*CcxCs*)	F_1(*CcxCs*)	0.538	—
CA-07	—	F_1(*CcxCs*)	0.52	0.48	0.00	*Hybrid* (*Cs,Cc*)	0.01	0.00	0.54	0.38	0.01	0.06	F_1(*CcxCs*)	F_1(*CcxCs*)	0.231	—

Cs: Castanea sativa; Cc: C. crenata; Cm: C. mollissima; F_1: first generation hybrid between *C. sativa* and *C. crenata;* F_2: second generation hybrid between *C. crenata* and *C. sativa; BC*(*Cs*): backcross of F_1 on *C. sativa; BC*(*Cc*): backcross of F_1 on *C. crenata.* Ho^3: Observed heterozygosis.

According to Camus (1929), 'Tamba' is a variety of Japanese chestnut that is grafted on 'Shiva', which the name is given to the wild Japanese chestnut. Consequently, the introduced seed lots may mainly be progenies of Tamba clones.

Identification of the genealogy of individuals included in the Clone Collection of Lourizán

We attempted to classify a large set of hybrids among three differentiated chestnut species, *C. sativa*, *C. cre-*

nata and *C. mollissima*, into genealogic classes using reference samples identified based on morphology and a reduced number (13) of isozyme *loci*. All of the three methods used had limitations for precise classification in cases in which F_2 individuals or backcrosses were present. For a precise determination of genealogical classes with genotype inspection, at least 10 diagnostic *loci* are necessary (Epifanio and Philipp, 1997), and in this study, the maximum number of diagnostic *loci* between *C. sativa* and *C. crenata* was found to be three, and two of these three were not completely diagnostic. The other two methods used, based on *STRUCTURE* and *NEWHYBRIDS* software, do not require diagnostic *loci*, but for a precise classification in cases in which backcrosses are present, at least 40 *loci* are necessary (Vaha and Primmer, 2006). This explains our finding that *NEWHYBRIDS* was not able to identify backcrosses in the studied sample of chestnut hybrids, although there is evidence they are present. As a consequence, it was possible to elaborate only a coarse classification and the three methods were combined to produce a more approximate classification using a series of considerations: genotype inspection and *STRUCTURE* were used to identify the ancestor species of each individual; threshold values of pure species reference samples identified with *STRUCTURE* and *NEWHYBRIDS* were used to classify other individuals with values under the threshold as F_2(*CcxCs*)-*BC*; and the genotypic class typical of F_2(*CcxCs*) is only present in F_2(*CcxCs*) genealogical classes. The results of the classification of individuals included in the Lourizán Clone Collection followed expectations: most individuals are hybrids between *C. crenata* and *C. sativa*; most clones of the Lourizán Clone Collection (*MB* + *EFAC*) are probably F_2(*CcxCs*) or backcrosses, the majority of which are probably to *Cs*; most *EFAC* clones are F_1(*CcxCs*). The Asiatic stands, which are the second generation of the two Asiatic species in the Iberian Península, contain a number of F_1(*CcxCs*) or F_2(*CcxCs*)-*BC*(*Cc*) individuals, indicating the considerable ability of the Japanese and European chestnut to hybridize.

The morphological traits that were used in the identification of pure species were found to work satisfactorily, but this morphological identification is not perfect, and the methods based on genotypes used here allowed us to classify some of them as hybrids. The presence of perfect type or collapsed glandular trichomes on the abaxial surface of the leaves is of particular interest for the identification of Japanese chestnuts of their hybrids. However, this is not a diagnostic morphological trait of hybrids between *C. crenata* and *C. sativa,* although a large number of the F_1(*CcxCs*) clones that are true F_1 plants according to historical data showed collapsed type trichomes. On further review of the relevant literature, we found a description of Japanese chestnut trees without glandular trichomes or exhibiting only a very low presence of glandular trichomes (Bagnaressi, 1956).

The Japanese and Chinese chestnuts from the Lourizán Research Centre (Pazo de Lourizán) included in this study (stands Granxa da Serra, Prado das Troitas and As Minas, with mainly Japanese chestnut; stand Agrovello, with mainly Chinese chestnut) are included in a list of unique singular trees that are protected due to their scarcity and historical significance (http://mediorural.xunta.es/areas/conservación/biodiversidade/arbores_senlleiras). In the present study, we detected a certain amount of hybridization with *C. sativa*. It is recommended that these stands be conserved by the plantation of new trees to compensate for mortality. The new plants can be obtained from open-pollinated progenies collected among pure *C. crenata* or *C. mollissima* trees, depending on the stand. The purity of these young individuals will be tested with molecular markers before plantation.

Acknowledgements

The lab work performed in this study was carried out at the Lourizán Research Centre in the period 1991-1995 with the support of the Spanish Programme for Agricultural Research. The main analyses conducted in this report were carried out recently using software developed during the last decade with the support of the project *PGIDIT07PXIB502128PR* (*INCITE*, Xunta de Galicia).

References

ANDERSON E.C., THOMPSON E.A., 2002. A model based method for identifying species hybrids using multilocus genetic data. Genetics 160, 1217-1229. Available in http://ib.berkeley.edu/labs/slatkin/eriq/software/software.htm#NewHybs.

BAGNARESSI U., 1956. Osservazioni morfo-biologiche sulle provenienze di castagno giapponese coltivate in Italia. Publicazone n. 3 Centro di Studio sul castagno Stazione Sperimentale di Selvicoltura di Firenze, 7-48.

BAZZIGHER V.G., 1981. Selektion *Endothia*-resistenter Kastanien in der Schweiz. Schweiz Z Forstwes 132(6), 453-466.

BOECKLEY W.J., HOWARD D.J., 1997. Genetic analysis of hybrid zones: number of markers and power of resolution. Ecology 78, 2611-1616.

BOLETÍN OFICIAL DEL ESTADO, 2007. Resolución del 25 de Octubre de 2007 de la Dirección General de Agricultura, por la que se publica la ampliación del Catálogo de materiales de base de diversas especies forestales de reproducción de las categorías identificada, cualificada y controlada. BOE núm. 27 del 13/11/2007.

CAMUS A.,1929. Les chataigniers. Monographie des genres *Castanea* et *Castanopsis*. París.

ELORRIETA J., 1949. El castaño en España. Ministerio de Agricultura, IFIE, Spain.

EPIFANIO J.M., PHILIPP D.P., 1997. Sources of misclassifying genealogical origins in mixed hybrid populations. J Hered 88, 62-65.

FALUSH D., STEPHENS M., PRITCHARD J.K., 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7(4), 574-578.

FERNÁNDEZ-LÓPEZ J., MIRANDA FONTAIÑA M.E., FURONES-PÉREZ P., 2008. Caracteres de selección en campo de clones de castaño híbrido (*Castanea crenata* × *Castanea sativa*) para la producción de madera. Cuad Soc Esp Cienc For 24, 39-43.

FERNÁNDEZ-LÓPEZ J., MIRANDA FONTAIÑA M.E., PEREIRA LORENZO S., 1995. Esquema de producción de materiales clonales forestales y frutales de castaño híbrido (*Castanea crenata* × *Castanea sativa*). ITEA 91(3), 149-156.

FERNÁNDEZ-LÓPEZ J., PEREIRA LORENZO S., MIRANDA FONTAIÑA M.E., 1992. Fog and substrate conditions for chestnut propagation by leafy cuttings. Symposium Proceedings Mass Production Technology for Genetically improved fast growing forest tree species. AFOCEL/IUFRO I, 379-383.

FERNÁNDEZ-LÓPEZ J., PEREIRA LORENZO S., MIRANDA FONTAIÑA M.E., 1995. Isozymes in the management of a Nuclear stock of chestnut hybrid clones. Sistemas y Recursos Forestales, fuera de serie nº 4, 131-136.

FERNÁNDEZ-LÓPEZ J., VÁZQUEZ-RUIZ DE OCENDA R., DÍAZ-VÁZQUEZ R., PEREIRA-LORENZO S., 2001. Evaluation of resistance of *Castanea* sp. clones to *Phytophthora* sp using excised shoots. For Snow and Res 76(3), 451-454.

FERNÁNDEZ-LÓPEZ J., MONTEAGUDO A.M., 2010. Genetic structure of Spanish wild populations of *Castanea sativa* as revealed by isozyme analysis. Forest Systems 19(2),156-169.

GALLÁSTEGUI C., 1926. Técnica de la hibridación artificial del castaño. Boletín de la Real Sociedad Española 26, 88-96.

GRAVES A.H.,1949. Key to chestnut species with notes on some hybrids. Annual Report of 14 th Annual Meeting of the Northern Grower Association. 95-107.

JAKOBSSON M., ROSENBERG N.A., 2007. CLUMPP: a cluster matching and permutation programme for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23, 1801-1806. Available in: http://rosenberglab.bioinformatics.med.umich.edu/clumpp.html

JAYNES R.A., 1964. Interspecific crosses in the genus *Castanea.* Silvae genetica 13(5),125-164.

LANG P., DANE F., KUBISIAK T., HUANG H., 2006. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. Molecular Phylogenetics and Evolution 43, 49-59.

MIRANDA-FONTAIÑA M.E., FERNÁNDEZ-LÓPEZ J., 2001. Genotypic and environmental variation of *Castanea crenata* × *Castanea sativa* clones in aptitude to micropropagation. Silvae genetica 3-4, 153-162.

MIRANDA-FONTAIÑA M.E., FERNÁNDEZ-LÓPEZ J., VETTRAINO A.M., VANNINI A., 2007. Resistance of *Castanea* Clones to *Phytophthora cinamomii*: testing and genetic control. Silvae Genetica 56(1), 11-21.

MOLINA F., VIEITEZ E., 1967. Defense du chataignier contre ses maladies en Espagne. Atti Convegno Internazionalle sul Castagno. Cuneo, 12-14 Ottobre, 1966. 107-114.

NASON J.D., ELLSTRAND N.C., 1993. Estimating the frequencies of genetically distinct classes of individuals in hybridized populations. Journal of Heredity 84-1, 1-12

PEAKALL R., SMOUSE P., 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288-295. http://www.anu.edu.au/BoZo/Genalex.

PRITCHARD J. K., STEPHENS M., DONNELLY P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945-959. Available in http://pritch.bsd.uchicago.edu/software

RINALLO C., BRUNO E., 1991. Foliar surface features of *Castanea sativa* Mill. Adv Hort Sci 5, 64-72.

ROSENBERG N.A., 2004. Distruct: a program for the graphical display of population structure. Mol Ecol Notes 4,137-138. Available in: http:// rosenberglab.bioinformatics.med.umich.edu/distruct.html

SALESSES G., RONCO L., CHAUVIN J.E., CHAPA J., 1993. Amélioration génétique du chataignier misse au point de tests d'évaluation du comportement vis-á-vis de la maladie de l'encre. L'arboriculture fruitiére 458, 23-31.

SCHAD C., SOLIGNAT G., GRENTE J., VENOT P., 1952. Recherches sur le chataignier a la station de Brive. Annales de L'amélioration des plantes 3, 369-458.

TAVEIRA-FERNÁNDES C., 1972. Aspects de l'amelioration du chataignier pour la résistance a la maladie de l'encre. In Actas III Congr Un Fitopat. Medit, Oeiras, Portugal. pp. 313-320.

URQUIJO-LANDALUZE P., 1944. Aspectos de la obtención de híbridos resistentes a la enfermedad del castaño. Bol Pat Veg y Ent Agrícola 13, 447-462.

URQUIJO-LANDALUZE P., 1957. La regeneración del castaño. Boletín de Patología Vegetal y Entomología Agrícola 22, 217-232.

VÄHÄ J-C., PRIMMER C.R., 2006. Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different number of loci. Mol Ecol 15, 63-72.

VIEITEZ E., 1966. Resistencia a *Phytophthora cambivora* y *P. cinnamomii* de algunas variedades de castaños. Anales del Instituto Forestal de Investigaciones y Experiencias I, 61-74.

VIEITEZ E.,1985. La influencia de Cruz Gallástegui en la mejora del castaño. Homenaje a Cruz Gallástegui Unamuno. Edited by Diputación de Pontevedra Pontevedra. pp.13-41.

WENDEL J.F., WEEDEN N.F., 1989. Visualization and Interpretation of Plant Isozymes. Isozymes in Plant Biology. Edited by DE Soltis, P Soltis P Dioscorides.