

## Geographic differentiation in adaptive traits of wild chestnut Spanish populations (*Castanea sativa* Miller)

J. Fernández-López\*, R. Zas, R. Blanco-Silva and R. Díaz

Centro de Investigaciones Forestales y Ambientales de Lourizán. Apdo. 127. 36080 Pontevedra, Spain

---

### Abstract

Flushing, spring frost damages and height were recorded in a provenance test including nineteen populations covering most Spanish chestnut distribution and were analysed to study the differentiation among wild populations. There was remarkable differentiation among populations in flushing time and height growth and Qst was higher for flushing and frost damage, and lower for height. The coefficients of quantitative differentiation among populations were similar to the values obtained for other autochthonous species. Spanish chestnut wild populations were classified in three groups: early flushing xeric populations from the Mediterranean, late flushing populations from northern mountains and intermediate flushing populations from Galician coast and Canary Islands. The observation of variability patterns indicated the importance of natural selection and a restricted gene flow as factors shaping the structure of wild populations in Spain. The use of local sources in plantations was recommended.

**Key words:** flushing time, quantitative differentiation, natural selection, provenance region.

### Resumen

#### Diferenciación geográfica de poblaciones españolas silvestres de castaño en caracteres adaptativos (*Castanea sativa* Miller)

Se analizaron datos de brotación, daños por heladas y crecimiento en altura de un ensayo de procedencias, con 19 poblaciones de gran parte del área de distribución española de *Castanea sativa*, con la finalidad de estudiar la diferenciación entre poblaciones. Se encontró una diferenciación importante en fechas de brotación y crecimiento. Los coeficientes de diferenciación cuantitativa entre poblaciones fueron similares a los valores obtenidos en otras especies autóctonas. Las poblaciones españolas de castaño silvestre se clasificaron en tres grupos: poblaciones xéricas mediterráneas de brotación temprana, poblaciones de brotación tardía de las montañas del Norte, poblaciones de carácter oceánico de brotación intermedia de la costa gallega e islas Canarias. Las pautas de variación geográfica encontradas indican que la selección natural junto con un flujo genético muy restringido fueron los factores determinantes de la estructura de las poblaciones. Se recomienda el uso de semillas de poblaciones locales en las plantaciones.

**Palabras clave:** brotación, diferenciación en caracteres cuantitativos, selección natural, región de procedencia.

---

### Introduction

Although genetic differentiation in adaptive traits is the expected pattern of variation between trees populations, the adaptation through large areas could be due to phenotypic plasticity, specially in introduced species. This could be the case of European chestnut (*Castanea sativa* Miller) in its western area. The broad range consisting in a scattered distribution in acid soils through South Europe and South West Asia, may have

resulted in some kind of differentiation among populations in adaptive traits. Drought and spring frosts are two strong selective factors causing differentiation in most temperate species (Eriksson and Ekberg, 2001; Morgenstern, 1996; Stern and Roche, 1974) and it is expected to be causal effects of natural selection in chestnut. The species is considered sensitive to spring frosts after flushing and tolerant to less than four dry months. Other evolutive force affecting differentiation among populations is the gene flow. A low rate of gene flow by seeds is expected due to nut dispersion by animals, but there is uncertainty about the importance of gene flow by pollen. Long distance gene flow would

---

\* Corresponding author: [ffina.cifal@siam-cma.org](mailto:ffina.cifal@siam-cma.org)  
Received: 02-03-04; Accepted: 15-02-05.

be expected under anemogamous pollination, that according with some authors occurs in dry environments (Breisch, 1995; Solignat and Chapa, 1975) counteracting effects of natural selection and, in consequence, decreasing geographic differentiation. Higher geographic differentiation is expected if entomophyllous pollination is preponderant as it was demonstrated recently (Manino *et al.*, 1991; Oliveira *et al.*, 2001).

However, considering that the actual distribution area of chestnut is in part the result of expansion by man, the phenotypic plasticity, more than adaptation, could explain its potential for occupying new areas. This could be especially true in Western Europe where the autochthonous origin of European chestnut is under discussion (Aira-Rodríguez and Rego, 1995; Fineschi *et al.*, 2000; García Antón *et al.*, 1990; Villani *et al.*, 1994). There are also strong evidences that the establishment of mono-specific plantations through very large areas of thousand hectares, in coppices and orchards, could affect natural patterns of genetic variability. Specifically these plantations could have avoided natural selection processes leading to differentiation. The extensive use of vegetative regeneration through successive coppicing hinders evolution and, in consequence, adaptation of the respective populations. The use of vegetative propagation by grafting of individuals selected for their nut or wood quality, in extensive monoclonal or few-clonal orchards, during hundreds or thousands years, as well as the movement of scions between regions, could affect the genetic variability of local wild stocks through gene flow and founder effects. All these factors could be dominant in shaping variability of actual wild populations. An additional factor affecting the genetic structure of most south western European forests is their origin in recolonization processes from reduced genetic stocks (Bradshaw, 2004). In the case of chestnut, this reduction could be still more significant due to the fact that, frequently, the remaining populations, are grafted orchards, constituted by only few clones. More knowledge about origins, genetic distance among wild and local varieties, the antiquity of domestication and gene flow is necessary to understand the structure and dynamics of chestnut wild populations.

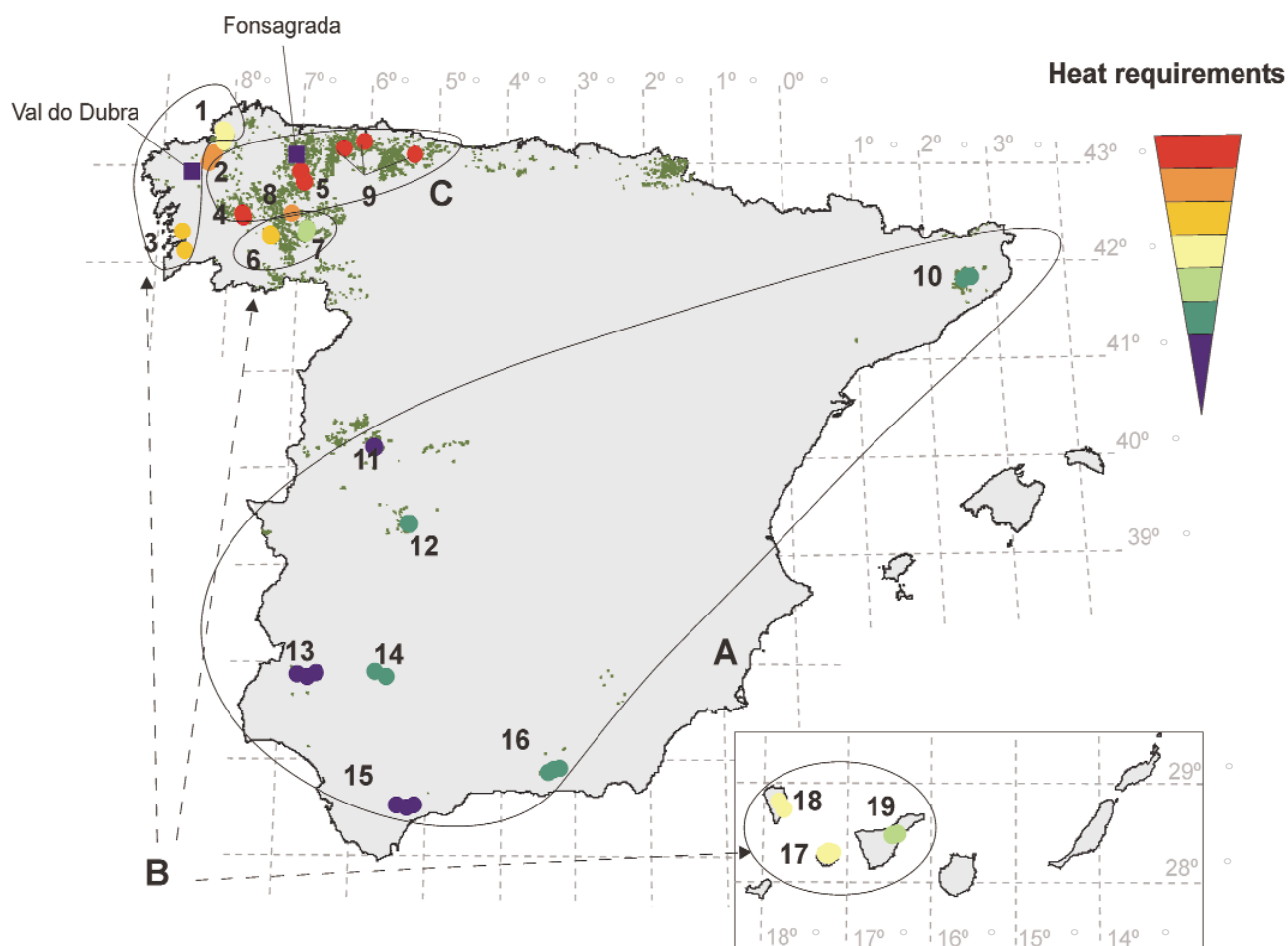
In Spain, pure chestnut stands have a discontinuous range, in several patches, occupying 137.657 ha (Figure 1). The largest continuous area is the North-western region, from Galicia to Navarra, occupying 70% of the total chestnut Spanish area. Climate in this

area is mainly Atlantic, increasing annual temperature oscillation from the coast to the mountains; besides in inland mountains of South Galicia and León temperature and summer drought are more extreme. Several discontinuous patches of chestnut grow under Mediterranean climate in Central West Spain (12%), and in the South (10.3%). Extreme drought conditions occur in Sierra de Aracena and Guadalupe with a drought period close to 4 months. These patches, occupying few thousand or hundred hectares, are separated by several hundred of kilometres and probably are reproductively isolated among them. Most areas occupied by mono-specific chestnut forests in Spain are coppices (36.0%) and orchards (37.5%), while high forest was very scarce in the past and is actually increasing. In the Northern area there are frequent processes of recolonization of abandoned agricultural lands and grafted chestnut orchards, while in the South natural regeneration is absent due to the intensive land use and perhaps to low survival of young seedlings under drought conditions.

The knowledge of the adaptive variation patterns of forest tree species of economic interest is essential for a sound management of their gene resources. The existence of geographical races with specific adaptations to local environments makes necessary the definition of provenance regions and breeding zones and the establishment of seed transfer rules (Buijtenen, 1992) and planning conservation activities. In fact, for the Spanish application of the European regulation on forest reproductive materials, the Directive 1999/105/CE (Anonymous, 1999; Anonymous, 2003), provenance regions common for several species, chestnut among them, were defined.

The best method to identify populations with similar adaptive characteristics in temperate regions is the study of relevant traits in provenance tests. The relevance of flushing as an adaptive trait is due to the sensibility of new sprouted leaves to late spring frost and to its relation with the length of the vegetative period and in consequence with the potential of growth, although other factors as drought avoidance could be involved in southern areas.

The main goal of the present research was to quantify the differentiation among Spanish wild chestnut populations and to detect the environmental factors involved in natural selection. We analysed flushing and height growth during three consecutive years in a provenance test planted in two sites. The final objective was the elaboration of recommendations for provenance use.



**Figure 1.** Chestnut populations sampled through the Spanish distribution. The scale of colours was elaborated from results of multiple means comparison test of flushing data from Fonsagrada site. Dots of the same colour indicated that means of the respective populations are statistically equal. The scale of colours goes from red, for later flushing populations, to blue for earlier flushing populations, indicating reducing heat requirements for flushing. Populations were grouped by flushing performance and stability. Group A: early flushing with high stability, group B intermediate flushing population with low stability and group C late flushing population with high stability (see Figure 3).

## Methods

### Plant material

In this text, the word *wild* was applied to chestnut populations regenerated from seeds, to differentiate them from grafted trees. Their origin could be from plantation or natural regeneration, from natural or naturalised origin, growing in mixed forest, coppice or high forest. Wild was used with the same meaning as the words *bravo* in Portuguese and Galician languages, or *silvatico* in Italian.

Populations were sampled through the Spanish chestnut distribution area (Fig. 1) following a hierarchic structure at two levels: the *population level* (P)

considered here as individuals potentially interbreeding, growing in a continuous area, with similar climatic conditions and the subpopulation level. Each *population* was represented by two to four *sub-populations* (SP) separated from 5 to 30 kilometres. The mean number of sub-populations per population was 2.7 and each sub-population was represented by the bulk mixture of seeds collected from 20 to 30 trees, separated among them at least 10 metres. Nineteen *populations* covering most of the Spanish distribution of the species were chosen for sampling. Geographic and climatic features of the populations included in the provenance test are presented in Table 1.

The Northern area was represented by nine populations covering most climatic variation: populations 1

**Table 1.** Geographical and climatic features of populations included in the provenance test. Climatic data from Allué-Andrade (1990)

Population	Natural area	Coordinates		Altitude (m)	Subpopulation number		Annual precipitation P (mm)	Mean temperature T (°C)	Mean temperature warmest month MTW (°C)	Summer precipitation SP (mm)
		Longitude	Latitude		Fonsagrada	Val do Dubra				
1	Rías Altas	8°10'	43°19'	20-115	4	—	866-871	12.1-14.3	16.9-18.7	25.0
2	Ordes	8°20'	43°06'	225-350	4	2	1,124-1,545	12-12.8	18.0-18.4	29.5
3	Rías Baixas	8°40'	42°11'	30-225	2	—	1,631-1,965	13.2-14.5	18.6-19.7	25.5
4	Meseta Lucense	7°50'	42°34'	660-675	2	—	1,342-1,552	9.9-11.9	17.9-18.9	20.5
5	Navia de Suarna	7°00'	42°57'	405-440	2	2	1,425-1,485	11.2-11.5	16.1-17.9	28.0
6	Val do Sil	7°26'	42°23'	420-610	3	1	597	13-13.6	21.8	16.0
7	Valdeorras	6°56'	42°26'	565-780	2	1	901	11.1-12.3	21.0	15.0
8	O Caurel	7°10'	42°38'	840	1	1	1,897	9.2	15.9	46.0
9	Asturias	6°08'	43°17'	60-630	3	1	1,126-1,306	11.2-13.8	17.3-18.8	36.0
10	Las Guillerías	2°27'	41°54'	610-950	4	1	655-859	10.8-12.1	20.7-21.1	47.0
11	Hervás	5°52'	40°15'	730-950	4	1	1,138	13.7-15.5	23.8	10.0
12	Guadalupe	5°20'	39°28'	930-1,100	4	1	783	12.8	24.2	3.0
13	Sierra de Aracena	6°34'	37°51'	540-920	3	1	981-1,104	14.2-16.6	25-25.8	3.0
14	Cazalla Sevilla	5°36'	37°53'	620	2	—	808	12.8	22.8	2.0
15	Serranía de Ronda	5°11'	36°32'	750-920	3	1	1,214	13.1-13.7	23.5	0.0
16	La Alpujarra	3°16'	36°56'	1,110-1,430	3	—	740	13-14.2	22.4	3.0
17	Gomera	17°12'	28°06'	600-1,100	3	—	695-725	13.1	—	1.0
18	La Palma	17°47'	28°30'	500-1,000	3	1	—	—	—	0.0
19	Tenerife	16°25'	28°21'	900-1,390	2	1	696	13-15	19.2	7.0

and 3 (Rías Altas and Rías Baixas) grow in a mild humid climate with a very low annual temperature oscillation; populations 5, 8 and 9 (Navia de Suarna, Caurel and Asturias) from Northern mountains, grow in cold humid climate; populations 2 and 4 (Ordes and Meseta Lucense) were in intermediate conditions between the two previous groups; populations 6 and 7 (Val do Sil e Valdeorras) develop in continental climate with summer drought and important temperature oscillation. Each discontinuous patch was represented by one population. Population 10 (Las Guillerías) is in North-East Spain, Girona; populations 11 and 12 (Hervás and Guadalupe) develop in Central West Spain; populations 13, 14, 15, 16 (Sierra de Aracena, Cazalla, Serranía de Ronda and Las Alpujarras) represent four separated patches in Andalucía; populations 17, 18 and 19 (La Gomera, La Palma and Tenerife) are naturalized populations introduced several hundreds years ago, in the Canary Islands. The number of sub-populations in each population is shown in Table 1, being 51 the total number of sub-populations.

Populations 1, 2 and 3 were pure chestnut stands or mixed forests, approximately with 30 years of age, growing in an area with strong natural regeneration since several decades. Populations 4, 5, 6, 7 and 13 were younger populations close to extensive grafted orchards,

probably offsprings of local grafted varieties. Populations 9, 10, 12 and 14 were high density coppices. Population 8 was an old orchard, perhaps a mixture of grafted and wild trees. Seed collection was made during autumn 1997. Seeds were conserved in the fridge several months and sown in March 1998 in 500 cc pots in the CIFA Lourizán nursery.

## Experiment description

During winter 1998/1999 a provenance test was planted at two sites in Northwest Spain under Atlantic climate conditions, with high precipitation and very low summer drought. Fonsagrada site ( $-7^{\circ}3'$ ;  $43^{\circ}11'$ ) is 750 m altitude, and was planted in November 1998, while Val do Dubra ( $-8^{\circ}41'$ ;  $43^{\circ}5'$ ) is 425 m altitude and was planted in March 1999. Mean annual temperature was similar in Fonsagrada than in Val do Dubra with values of 10.4 and 10.0°C, respectively. Annual precipitation was high in Fonsagrada and very high in Val do Dubra with mean annual values of 1,299 and 2,304 mm respectively.

The experiment was planned as a random complete block design with one-tree plot, although there was a certain unbalance due to the poor conservation of some seed lots. At Fonsagrada site, there were 25 blocks and

19 populations, while at Val do Dubra there were 20 blocks and 13 populations (Table 1). Total number of planted seedlings were 1,168 at Fonsagrada and 292 at Val do Dubra.

## Variables description

The traits assessed were apical and lateral flushing (TFL, LFL), spring frost damages (FROST), survival and height (H). The variable acronym is followed by 99, 00 or 01 for the respective data recorded in 1999, 2000 and 2001 and by A or B for the first and the second flushing record within each year.

Flushing was recorded following an eight points scale, modified from (Solignat and Chapa, 1975): 1, dormant bud; 2, bud initiate swelling; 3, bud flushed, green leaves shorter than brown scales; 4, green scales longer than brown ones; 5, nerves of leaves are evident; 6, shoot length less than 5 cm; 7, shoot length between 5 and 10 cm; 8, shoot length longer than 10 cm. The scale was extended over stage 4, in which a plant is considered flushed, to improve fulfilment of parametric conditions for analysis of variance and because the use of the expanded scale in the analysis of single data scores improves the estimation of genetic variability in flushing (data not reported).

Records of flushing stage of the most distal bud (TFL), and of most frequent stage among lateral buds (LFL) were made three consecutive years (1999, 2000, 2001). Two data records were assessed each spring (a, b), at each plantation, separated from 8 to 21 days. In Fonsagrada, data records were made 8 and 24 April 1999, 20 and 28 March 2000; 2 and 12 April 2001. In Val do Dubra records were made 6 and 27 April 1999, 20 and 28 March 2000 and 28 March and 6 April in 2001. Flushed buds are sensitive to frosts after stages 3 or 4 (Breisch, 1995). Frosts damages (FROST) were assessed following a four-point scale, depending on the percentage of buds damaged by frosts in a plant: 1, from 0 to 25%; 2, from 25 to 50%; 3, from 50 to 75% and 4, more than 75%. Survival was registered as a binary data (0,1) with value 0 for dead plants. Total seedling height (cm) was measured at the end of the first and second year after plantation (H99, H00). Height at the end of the second year in Fonsagrada was discarded due to damages caused by deer browsing. Coefficients of variation were calculated at subpopulation level for the flushing score and total height as an estimation of variability within stands (Bastien and Alia, 2000).

## Data analysis

Flushing scores are typical rank variables that can be analysed with the normal theory of statistical tests if including more than five categories. Differences between genetic entries for flushing time were studied using different types of variables, being the analysis of single date scores one of them. This is a cheap method that allows an efficient detection of differences between genetic entries if records are taken when 50% of the trees have flushed (Baliuckas *et al.*, 1999; Jermstad *et al.*, 2001). In this experiment, raw flushing scores were used for the analysis of each date data, while for the joint analysis of years and sites standardised data were used to remove the effect of different means and variances across years and sites.

For the estimation of differences among populations, a statistical data analysis was performed with the PROC GLM (SAS-Institute, 1999) for flushing traits, frost damages and height growth with the following models:

For separate analysis of each site and date:

— Model 1:

$$X_{ijkl} = \mu + P_i + SP_{j(i)} + B_k + P*B_{ik} + \epsilon_{l(ijk)}$$

For separate analysis of each site combining three years data of flushing:

— Model 2:

$$X_{ijklm} = \mu + P_i + SP_{j(i)} + B_k + P*B_{ik} + Y_m + P*Y_{im} + SP*Y_{jm(i)} + \epsilon_{l(ijkm)}$$

For the joint analysis of two sites and the three years:

— Model 3:

$$X_{iklmn} = \mu + P_i + B_{k(n)} + P*B_{ik(n)} + Y_m + P*Y_{im} + S_n + P*S_{in} + \epsilon_{l(ikmn)}$$

where  $X_{ijkl}$ ,  $X_{ijklm}$ ,  $X_{iklmn}$ , were the observations in individual  $l$ ,  $\mu$  was the global mean,  $P_i$  the random effect of population  $i$ ,  $SP_{j(i)}$  was the random effect of subpopulation  $j$  within population  $i$ ,  $B_k$  the random effect of block  $k$  within site  $n$ ,  $P*B_{ik}$  was the random effect of population by block within site interaction,  $Y_m$  and  $S_n$  were the fixed effects of year  $m$  and site  $n$ ,  $P*Y_{im}$  and  $SP*Y_{jm(i)}$  were the random effects of the population-year and subpopulations-year interactions, and  $\epsilon_{l(ijk)}$  was the random error. At site Val do Dubra,  $SP_{j(i)}$  was removed from the model due to the lower number of stands representing each population within this site. The RANDOM statement of GLM was used for the estimation of square means, which were used to calculate components of variance, equating to correspondent

mean squares. The total variance was divided into their components:

$$\text{Model 1: } V_T = V_P + V_{SP} + V_{P*B} + V_e$$

$$\text{Model 2: } V_T = V_P + V_{SP} + V_{P*B} + V_{P*Y} + V_{SP*Y} + V_e$$

$$\text{Model 3: } V_T = V_P + V_{P*Y} + V_{P*S} + V_{P*B} + V_e$$

where  $V_P$  was the variance among populations,  $V_{SP}$  the variance among sub-populations within populations,  $V_{P*B}$  the variance the population\*block interaction,  $V_{P*Y}$  and  $V_{P*S}$  were the variances due to interactions population-year and population-site and  $V_e$  was the error variance.

For grouping similar performance populations, ranks means of flushing within each site were plotted against the measure of rank stability among years  $S_{ii}$ , proposed by (Hühn, 1979) and calculated as follows:

$$S_{ii} = \frac{2 \sum_{j < j'} |r_{ij} - r_{ij'}|}{n(n-1)}$$

where  $r_{ij}$  is the rank of the  $i^{\text{th}}$  population at age  $j$  and  $n$  are the number of measurements. The relative importance of plasticity for populations was estimated from stability ( $S_{ii}$ ) of provenance performance among years in each site and from the visual comparison of both sites plots.

The differentiation among populations in quantitative adaptive traits,  $Q_{ST}$ , was calculated by the measure proposed by Spitze (1998) and Kremer (1997):

$$Q_{ST} = \frac{\sigma_{GB}^2}{\sigma_{GB}^2 + 2\sigma_{GW}^2}$$

being  $\sigma_{GB}^2$  the genetic variance among populations and  $\sigma_{GW}^2$  the genetic variance within populations. Estimates were calculated separately for each site and year, for each site combining the three years, and for the joint analysis of both sites. At Fonsagrada, the variance component among populations was subdivided in variance among populations,  $Q_{ST}[P]$ , and the variance among sub-populations within populations  $Q_{ST}[SP]$ . For example for the combined analysis of both sites for height:

$$V_T = V_P + V_{P*S} + V_S + V_{B(S)} + V_e$$

$$\sigma_{GB}^2 = V_P \quad \sigma_{GW}^2 = h^2_i (V_{P*S} + V_e)$$

$$Q_{ST} = \frac{V_P}{V_P + 2h^2_i (V_{P*S} + V_e)}$$

As individual heritability ( $h^2$ ) could not be estimated due to the lack of family structure the values used were

0.20 for flushing and frost damages and 0.4 for height values, obtained in *C. sativa* progeny tests (Fernández-López, personal communication; Pliura and Eriksson, 2002). Correlation between traits was calculated using original data.

Subsequently, the relationship between population performance and climatic and geographic data in the origin was studied. Prior to the analyses, flushing data were standardized at each test site in order to homogenize variance. A principal component analysis was done using scalars reflecting geographic and climatic variables at origin. So, linear regression analyses were done between height increment, frost damages, flushing and coefficient of variation of flushing and geographic data (latitude, longitude, altitude and coast distance), the climatic ones (pluviometric, thermal and thermo-pluviometric factors) obtained from Allué (1990) but temperature and precipitation variables were corrected by altitude differences and the principal component variables. Canary island provenances were not considered in these analyses, because existing pluviometric data do not reflect the real water availability by trees, as horizontal precipitation was not estimated.

The observation of results on flushing rankings and their stability among years (Fig. 3) as well as the relationship between flushing and variables related to drought suggested to cluster populations in three regions. Regions A and C included early and late flushing stable populations, respectively, while region B included intermediate and unstable flushing populations. Flushing, frost damages and height were re-analysed again grouping populations into these regions, using the model:

Model 4:

$$X_{iklmn} = \mu + R_o + P_{i(o)} + S_n + B_{k(n)} + Y_m + R*S_{on} + P*S_{in(o)} + R*Y_{om} + P*Y_{im(o)} + \epsilon_{l(ikmno)}$$

where  $R_o$  was the region factor, and  $R*S_{on}$ ,  $R*Y_{om}$  were the region-site and region-year interactions, respectively.

## Results

The mortality after three years was very low in both sites with values of 5.5% in Fonsagrada and 1.7% in Val do Dubra. Differences of the percentages of variance due to populations between the two consecutive assessments of phenology within a year, were similar. This indicated that the estimation of differentiation among populations,

using only one date record, was not very sensitive to the stage of development of plants. Percentages of variation among populations were similar for terminal and lateral buds in both plantations. Terminal buds flushed earlier than lateral ones in all studied conditions, indicating an acrotonic flushing. The phenomena was most evident in spring 1999. The difference TFL-LFL was lower in Fonsagrada than in Val do Dubra site with values of 0.64 and 0.74, respectively.

Damages caused by frost during flushing time, occurred during the first two springs at both sites, when temperature dropped below 0°C and flushing score was higher than 4. At Fonsagrada, high frost damages were recorded on April 24, 1999 (FROST99B) after four consecutive frost days (−1.8°C) when mean flushing stage was 5.5. Damages occurring in frost days, but occurring with low flushing scores were very light due to low flushing stages and to the short frost exposition. For example on the 20.03.2000 (FROST00A) temperature fall to −1.8°C when mean flushing was 3.5 and very low damages occurred. Frost damages in Val do Dubra were significant on the 27.04.1999 (FROST99B) after temperature drop to −0.3°C and mean flushing stage was more advanced than 4.

The phenotypic correlation between frost damage and flushing scores was significant at both sites, in 1999, being specially high in Val do Dubra (+0.61). Flushing and height were negatively correlated with moderate values at both sites (−0.23 in Fonsagrada and −0.38 in Val do Dubra). In Val do Dubra, where most severe frost damages occurred, survival and frosts were moderately correlated (−0.23).

## Differentiation among populations for flushing

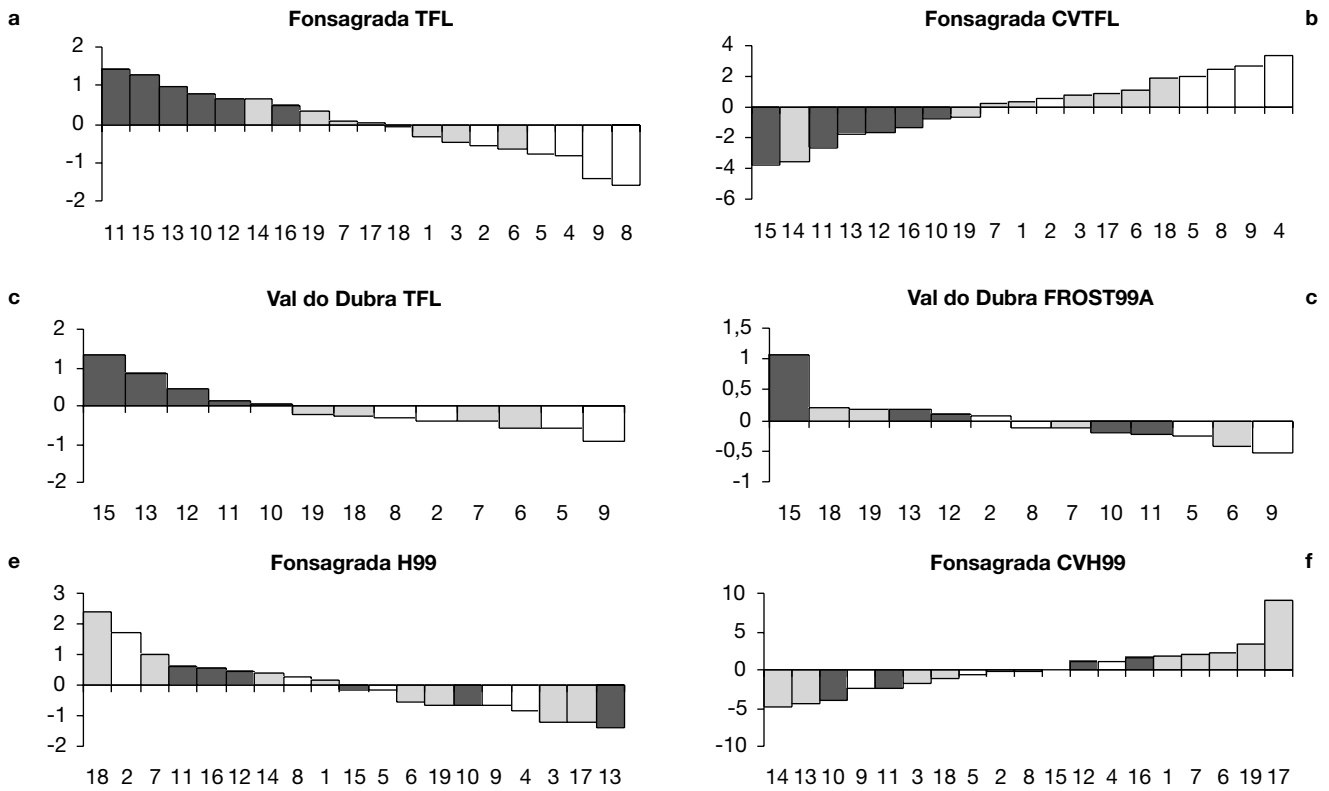
Results of the ANOVAs indicated that differentiation among populations for flushing was very important specially for the joint analysis of the three years by site (Model 2, Table 2), as well as for the joint analysis of the three years and both sites (Model 3, Table 2). Variance among populations for flushing of terminal buds (expressed as percentage of total variance and not reported in tables) accounted for 11 to 28% in Val do Dubra and for 15 to 30% in Fonsagrada, in the individual analysis (Model 1). In the joint analysis of the three years the percentage of variance explained by populations was 10.2 and 27.9% in Fonsagrada and Val do Dubra, respectively (Model 2), while in the joint analysis of the two sites and the three years, accounted for 15.3%. In Fonsagrada, the variance among populations was subdivided in the two components  $V_p$  and  $V_{sp}$ . Variance among subpopulations within populations was very important in the individual analysis, accounting for a percentage of variance almost as important as variance among populations. However, in the joint analysis of the three years the subpopulation component was insignificant, while there was a moderate interaction population x year.

Rankings of populations made with data recorded in terminal and lateral flushing were similar but terminal flushing was selected for classification due to the higher objectivity of the assessment. Deviations of population mean values from the global site mean are represented in Fig. 2. Earlier flushing populations

**Table 2.** Results for the joint analysis of flushing records of three years for each site and for the joint analysis of both sites presented as mean squares and level of significance factor in the model and significance level of factors (•, \*, \*\*, \*\*\* means F-statistics of sources of variance are significative at the levels 10% , 5% , 1% , 1%)

Variable	Site	NSP	P	SP	B	Y	P*B	P*Y	SP*Y	S	P*S	ε
TFLst	Fonsagrada	54	16.93***	1.52***	9.59***	0.52	0.96***	4.73***	0.95	—	—	0.72
	Fonsagrada	14	9.33***	—	5.95***	2.25*	—	1.65***	—	—	—	0.73
	Val do Dubra	14	18.93***	—	1.69***	0.08	—	0.87	—	—	—	0.71
	Both sites	14	23.42***	—	3.54***	1.85*	1.00***	1.63***	—	3.69*	2.59***	0.60
FROST99B	Fonsagrada	54	0.57***	0.74***	0.24	—	0.14	—	—	—	—	0.22
	Val do Dubra	14	3.23***	—	0.59	—	—	—	—	—	—	0.57
H99	Fonsagrada	54	2,234.60***	1,372.43***	636.65***	—	241.67	—	—	—	—	249.68
	Val do Dubra	14	2,376.68***	—	216.36	—	—	—	—	—	—	210.42
	Both sites	54	2,894.35***	—	437.56***	—	—	—	—	9,887.81***	1,645.07***	283.22
H00	Val do Dubra	14	2,163.05***	—	229.44	—	—	—	—	—	—	309.64

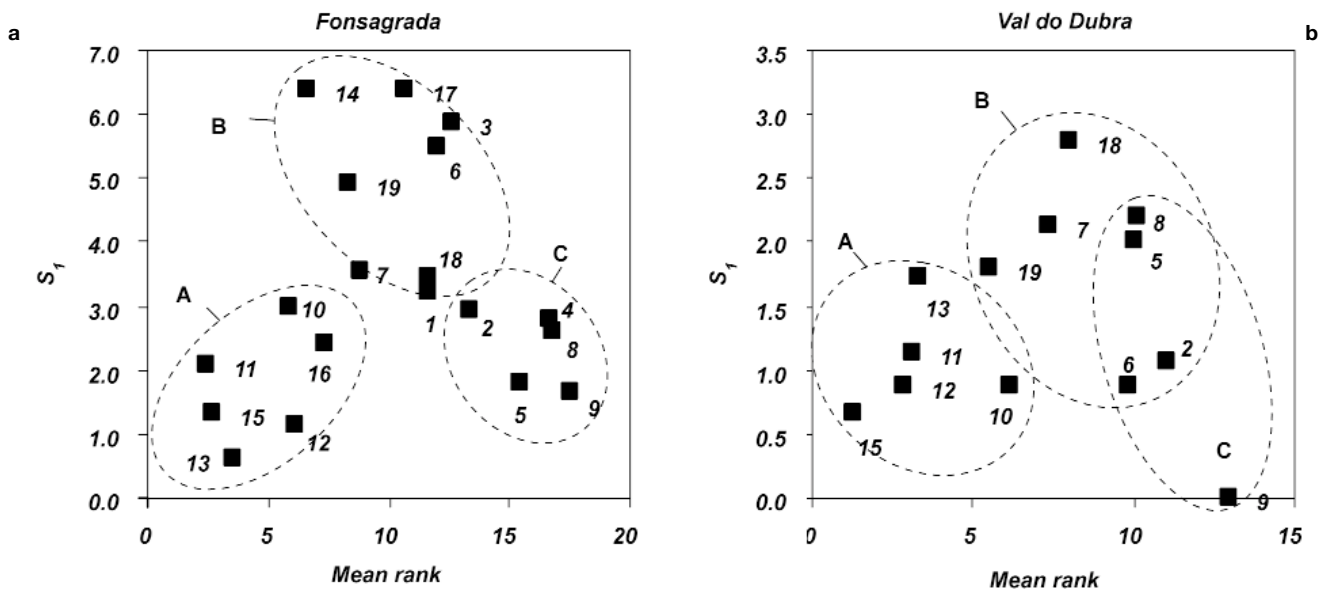
NSP: number of subpopulations. SP: subpopulations. P: populations. B: block. Y: year. S: site. ε: error. TFL<sub>st</sub>: flushing of terminal bud standardised for each year and site. FROST99 and FROST00 frost damage in the year 1999 and 2000 respectively. H99 and H00 total height at the end of the year 1999 and 2000 respectively.



**Figure 2.** Deviations of mean population values from global site mean. Values of flushing were the mean of three years after standardization. Decreasing order was used for flushing, frost, and height; increasing order for the coefficient of variation. Bar colours denote the three main population groups: Group A as black, Group B as grey and Group C as white (see Figure 3).

were all Mediterranean ones from the North-East, Central-West and South (10, 11, 12, 13, 14, 15 and 16). The populations 11, 13 and 15 were always the earliest

while 12, 14, 16 and 10 flushed a little later. The population 14, from Cazalla, in Sevilla, presented marked ranking changes among years (Fig. 3). All populations



**Figure 3.** Classification of populations by mean ranks of flushing and the average rank differences among years ( $S_1$ ). Populations were grouped by performance.



from the North-western area flushed later than southern ones although there were variations among them (Figs. 1-3). The latest flushing populations was 9 followed by 2, 4, 5 and 8, all they originated from Northern mountains and high Galician plateau. Intermediate flushing populations were those from low altitude in the Galician coast (1 and 3), from the Sil Valley (6, 7), and from the Canary Islands (17, 18 and 19). Most populations from the intermediate flushing group (3, 6, 7, 17, 18 and 19) were unsteady among years (Fig. 3).

### Differentiation among populations for frost damages

The ANOVAs for 1999 frost damages, indicated significant influence of the factor population, that in the case of Val do Dubra accounted for 14.9% of the variation (see square sums in Table 2).

The highest frost damages in this site were in population 15 followed by populations 18, 19 and 13

while lower damages occurred in plants of population 9 followed by plants of populations 6, 5, 11 and 10. Populations 2 and 8 were in an intermediate position (Fig. 2d).

### Differentiation among populations for height growth

The differentiation among populations was also important for growth, accounting between 24 to 16.7% of the variance. Data from Fonsagrada (Table 3) indicated that the variance among subpopulations was more important than variance among populations. In the joint analysis of both sites the component for populations is lower (8.5%) due to a significant interaction population  $\times$  site accounting for 19.4% of variance. Observation of mean height growth values did not allow to recognise a clear geographic pattern (Fig. 2e).

The ANOVAs of coefficients of variation calculated at subpopulation level for flushing and height indicated

**Table 3.** Values of differentiation among populations [ $Q_{st}(P)$ ], subpopulations [ $Q_{st}(SP)$ ] and regions [ $Q_{st}(R)$ ] for flushing, frost damages and height growth

Trait	Site	Year	Variable	NSP	$Q_{st}(R)$	$Q_{st}(P)$	$Q_{st}SP$
Flushing	Fonsagrada	1999	TFL99A	All	—	0.34	0.09
		2000	TFL00A	All	—	0.12	0.22
		2001	TFL01A	All	—	0.31	0.26
		All	TFL <sub>st</sub>	All	—	0.16	0.02
		All	TFL <sub>st</sub>	Common	—	0.25	—
	Val do Dubra	1999	TFL99A	All	—	0.27	—
		2000	TFL00A	All	—	0.42	—
		2001	TFL01A	All	—	0.42	—
		All	TFL <sub>st</sub>	All	—	0.49	—
	Both	All	TFL <sub>st</sub>	Common	—	0.33	—
					0.36	0.00	—
Frost damages	Fonsagrada	1999	FROST99B	All	—	0.00	0.46
			FROST99B	All	0.05	0.07	—
	Val do Dubra	1999	FROST99B	All	—	0.23	—
			FROST99B	All	0.04	0.34	—
Height	Fonsagrada	1999	H99	All	—	0.06	0.17
	Val do Dubra	1999	H99	All	—	0.18	—
		2000	H00	All	—	0.13	—
	Both	1999	H99	Common	—	0.10	—
					0.08	0.03	—

NSP: number of subpopulations. R: grouping of populations by performance. SP: subpopulations. P: populations. TFL99A, TFL00A, TFL01A: first measurement of flushing of terminal bud for the year 1999, 2000 and 2001 respectively. TFL<sub>st</sub>: flushing of terminal bud standardised for each year and site. FROST99 and FROST00 frost damage in the year 1999 and 2000 respectively.; H99 and H00 total height at the end of the year 1999 and 2000 respectively. —: indicates that the respective factor was not included in the model.

an important differentiation among populations (data not presented). Coefficients of variation for flushing were low in populations 10 to 16, while these coefficients were specially high in populations 4, 5, 8 and 9 (Fig. 2b and 2f).

### Coefficient of differentiation in quantitative traits

The values of the quantitative coefficients of differentiation for different traits and sizes among populations were reported in Table 3. For flushing Qst values estimated for each year and site ranged from 0.43 to 0.57 [Qst(P)+Qst(SP)] in Fonsagrada and from 0.27 to 0.42 in Val do Dubra. When estimation of Qst was made with three years data for each site, the value decreased to 0.16 in Fonsagrada and increased a little in Val do Dubra to 0.49. For the shared populations between both sites and three years, the value of Qst was 0.33. The Qst for frost damages were 0.46 and 0.23 for year 1999 in Fonsagrada and Val do Dubra. Finally for height growth Qst was between 0.13 and 0.18 and decrease to 0.10 for the analysis of common populations in two sites.

### Relationship with environmental factors

The four principal components of four geographical and nine climatic data in the origin explained 82% of the total environmental variability. The first component explained the North-South variation in *drought* (37% of the total variability); the second

component explained the *winter cold* (23% of the total variability); the third and four components explained the *oceanic tendency of climate* (13% and 9% of the total variability).

The regression of the flushing scores and growth on climatic and geographic parameters in the origin indicated an important relation of flushing and its coefficient of variation within subpopulations with latitude, with the mean temperature of the warmest month and with the summer precipitation as well as with the first principal component PRIN1 that summarises several climatic variables related with drought (Table 4). Populations at higher latitudes flushed later and had higher coefficients of variation for flushing than southern ones.

### Regionalization of Spanish wild chestnut populations

Results of the analysis with model 4 (Table 5) indicate that grouping populations into regions improves the model explanation for flushing and that most variation was among regions. Trends of variation observed for vigour were different because most variation was within populations, although there was a slight differentiation between regions.

## Discussion

### Differentiation among populations

The differentiation among populations in flushing time and height growth at early ages was remarkable in this

**Table 4.** Regressions of phenological variables on climatic and geographical parameters at origin

Site	Variable	LAT			TMC			PE			PRIN1		
		s	R <sup>2</sup>	P	s	R <sup>2</sup>	P	s	R <sup>2</sup>	P	s	R <sup>2</sup>	P
Fonsagrada	TFL <sub>st</sub>	–	0.66	**	+	0.63	**				+	0.68	**
	CVTFL	+	0.77	***	–	0.55	*	+	0.53	*	–	0.66	**
Val do Dubra	TFL <sub>st</sub>	–	0.85	***							+	0.70	*
	CVTFL	+	0.81	**	–	0.64	*	+	0.64	*	–	0.71	*
Both	TFL <sub>st</sub>	–	0.74	**							+	0.69	*
	CVTFL	+	0.68	*									

CVTFL: coefficient of variation of terminal bud flushing. LAT: latitude. TMC: mean temperature of the warmest month. PE: summer precipitation. PRIN1: first principal component. s: sign. R<sup>2</sup>: coefficient of multiple determination. P: \*, \*\*, \*\*\* means F-statistics of sources of variance were significative at the levels 5% , 1% , 1%. See other abbreviations in previous tables.

**Table 5.** Results for the joint analysis of both sites gathering populations to regions. For flushing the factor year and their interactions with other factors were added. Results expressed as mean squares and levels of significance represented by •, \*, \*\*, \*\*\* for 10%, 5%, 1%, 1%

Variable	Site	R	P	S	B	Y	R*S	P*S	R*Y	P*Y	ε
TF <sub>st</sub>	Both	130.99***	4.60***	2.76*	4.07***	1.80•	6.13***	2.28***	1.91*	1.48**	0.72
FROST99B	Fonsagrada	0.12*	0.05*	—	0.04*	—	—	—	—	—	0.03
FROST99B	Val do Dubra	3.97**	3.17***	—	0.58	—	—	—	—	—	0.57
H99	Both	6,164.40***	2,369.10***	9,701.30***	437.55***	—	899.08*	1,729.16***	—	—	222.02

R: grouping of populations by performance. P: populations. S: site. B: block. Y: year. R\*S, P\*S, R\*Y and P\*Y the interactions region × site, population × site, region × year and population × year. ε: error. TFL<sub>st</sub>: flushing of terminal bud standardised for each year and site; FROST99 and FROST00 frost damage in the year 1999 and 2000 respectively.; H99: total height at the end of the year 1999.

experiment. However important differences among both types of traits were found. The differentiation of subpopulations within populations was much more important for height than for flushing. While the low values of the interactions population × site and population × year indicated a very high stability across sites and years for flushing, height was very affected by site conditions and the interaction population × site, as can be observed from the square sums in Table 2. The high genetic control of flushing is well known. The interactions P\*S and P\*Y could be explained by differences among populations in heat and cold needs accumulated for flushing.

The significant relationship between flushing and latitude, mean annual temperature of the warmest month and summer rainfall is due to a clinal variation South-North of needs of heat for flushing (Fig. 1), to an increment of resistance to frosts and of variability within populations following the same cline. The geographically extreme populations, i.e. the Southern population 15 in Serranía de Ronda and the Northern population 9 were the extremes in distance and also in flushing rankings through the S-N cline. There was not apparent differentiation in vigour along this cline. A second cline could be considered in the North, from population 1 by the sea level and minimum annual temperature oscillation, to population 9 in Asturias (data not reported). Through this second cline there was also increasing needs of heat for flushing and a certain increase of resistance to frosts.

Among the southern group of populations, 16, 14 and 12 flushed later than populations 11, 13 and 15. A possible explanation is a higher action of natural selection in these last three populations due to natural regeneration, while within populations 12 and 14, treated by shoot stump regeneration, the effects of natural selection may be minimum. Regarding population 16, in La Alpujarra, is a population with artificial irrigation since hundred years. In the North area, populations from the Sil Valley,

growing in summer drought conditions, i.e. population 7 flushed earlier than other neighbour populations, i.e. population 8 from Caurel, despite of the short distance and small altitude difference. Climate changes suddenly from the Val do Sil to Caurel, with decreasing temperature and increasing rainfall. The respective populations behaviour followed the described patterns of Mediterranean and Northern mountains populations, respectively.

Clinal variation is the result of natural selection by gradually changing environment usual in widely distributed wind pollinated species (Morgenstern, 1996). However described patterns of variation for the North of Spain, with specific adaptations due to natural selection and evidence of low or absent gene flow, are typical of ecotypic variation or small disjointed populations (Eriksson and Ekberg, 2001). Differentiation patterns within the Northern area indicates some restriction of gene flow, typical of an entomogamous species, as was determined by (Manino *et al.*, (1991) and Oliveira *et al.* (2001). The differentiation between populations 7 *versus* 8, and of 1 *versus* 2, could be explained by different selection pressures and a low or absent gene flow.

The observed patterns of variation indicate that natural selection shaped the actual structure of chestnut Spanish populations. The correlation between flushing, frost damages and survival indicated that frost is a relevant factor acting in natural selection. Thus, directional selection favouring late flushing seemed to be caused by frost damages in northern populations.

Meanwhile in southern and central populations it is establish the hypothesis of drought as the factor causing differentiation through directional selection. However in this experiment it was not possible to obtain any indication about the mechanism of the differential effect of drought in the populations due to the conditions of both trial sites. The lower coefficient

of variation within stands for flushing and height of southern populations indicate that the intensity of natural selection is higher in southern populations. In fact the experience obtained from other *C. sativa* provenance test in Mediterranean dry climate, showed a high mortality of populations from humid areas during the first years, which could indicate a strong directional selection caused by drought.

### Regionalization of Spanish wild chestnut populations

The existing differentiation for vigour was due to the higher growth of plants of some populations from the Galician coast and the Canary Islands. Climatic conditions in these areas are characterised by the low annual temperature oscillation and a high relative humidity during most of the vegetative period, both favourable to chestnut growth. Similar results were obtained for a population from the Galician coast, in a phytotron and a field experiment comparing six European populations throughout the European area [(Pliura and Eriksson, 2002; Fernández-López *et al.*, (in revision)]. Among the populations analysed in this study was also one population from the Galician coast, which showed the highest growth.

As summary, Spanish chestnut wild populations were classified in three groups. Xerophyllous Mediterranean populations from the South, Central West and North East Spain, from the hottest and driest climate, which were early flushing populations with lower variability within stands and were included in region A (Figs. 1 and 3). Genuine Atlantic populations, from Northern mountains and high plateau, growing in the coldest and the most humid climate, which showed late flushing and higher variability within stands, and were grouped in region C. Mesophyllous populations of intermediate flushing, with pronounced unsteadiness of flushing ranks among years and high vigour, which were the populations from North Galician coast and Canary Islands where the climate is characterised by the low annual temperature oscillation and absence of drought.

The coefficients of quantitative differentiation between populations were similar to coefficients obtained in the autochthonous broadleaves species *Quercus petraea* (Kremer *et al.*, 1997) for flushing and height growth.

The described geographic differentiation suggest that the adaptation of chestnut Spanish populations is

due to past events of natural selection rather than to phenotypic plasticity. However, phenotypic plasticity could not be evidenced in this experiment due to the fact that both sites enjoy similar Atlantic conditions. Other conclusion is that adaptation played an important role in western European chestnut area with independence of the management practices impeding evolution, as grafting and coppicing. This results also indicates that, after recolonization processes, the geographic adaptive structure is recovered. Perhaps the domestication degree of chestnut orchards or the scions movement between zones was not as important in the past as it was usually considered. Some of the studied populations were descendant from grafted trees (i.e. 4, 5, 6 and 7) and these results indicate that most grafts might have been selected in local wild populations. This conclusion agrees with the results obtained by an isozyme study comparing wild chestnut stands and orchards in the same area (Blanco-Silva and Fernández López, under revision). The differentiation patterns in flushing were similar to the observed in other species as *Quercus petraea* (Ducousso *et al.*, 1996) and *Populus tremula* (Alba-Monfort, 2000) with Southern populations flushing before than Northern ones.

### Application to management

This information indicates the convenience of the use of local materials in plantations as well as helps in the delimitation of breeding zones, provenance regions and in conservation planning (Fernández-López and Alía, 2003). The use of local populations safeguard adaptation to local climate while gains in growth (and perhaps for quality traits) could be achieved by the selection within each provenance.

### Conclusion

1. A remarkable differentiation among Spanish populations in flushing time and an important differentiation within populations for growth was found.
2. Spanish chestnut wild populations were classified in three groups. Early flushing populations with lower variability within stands from xerophyllous Mediterranean areas; late flushing mesophyllous populations with higher variability within stands and more resistant to spring frosts from Northern mountains and high plateau; intermediate flushing populations, with pro-

nounced instability of flushing ranks among years and higher vigour, from North Galician coast and Canary Islands.

3. The results indicate the importance of natural selection by frosts in Northern populations.

4. The patterns of variation indicated the convenience of using local materials in plantations and served to the delimitation of breeding zones if breeding is considered.

## Acknowledgements

The provenance test was developed within the Spanish project SC99-036 financed by the National Programme of Agricultural Resources and Technologies. The authors thank to all the collaborators from National and Natural Parks in seeds collection. Special thanks to Sonia Martín from the Ministry of the Environment for her help in contacts during collection, to Miguel Jamardo Figueira and to Irene Martín in charge of field work, data records and files. The map of chestnut distribution was yield up by DGCN of the Spanish Ministry of the Environment.

## References

- AIRA-RODRÍGUEZ M.J., REGO P.R., 1995. Paleobotanical data from northern Portugal (Baixo Minho) from pollen analysis and fossil seeds. *Lagasalia* 18(1), 25-38.
- ALBA-MONFORT N., 2000. Variabilidad genética de *Populus alba* L. mediante caracteres isoenzimáticos y fenotípicos. Aplicación a la selección y conservación de recursos genéticos. Tesis doctoral. Silvopascicultura. Universidad Politécnica, Madrid, p. 144.
- ALLÚE-ANDRADE J.L., 1990. Atlas fitoclimático de España. Taxonomías. Ministerio de Agricultura, Pesca y Alimentación, Instituto Nacional de Investigaciones Agrarias, Madrid, p. 222.
- ANONIMOUS, 1999. Directiva 1999/105/CE del Consejo de 22 Diciembre sobre la comercialización de materiales forestales de reproducción. Diario Oficial de las Comunidades Europeas, L 11/7-L11/40.
- ANONIMOUS, 2003. Real Decreto 289/2003, de 7 de Marzo, sobre comercialización de los materiales forestales de reproducción. BOE 58, 9262-9293.
- BALIUCKAS V., EKBERG I., ERIKSSON G., NORELL L., 1999. Genetic Variation Among and Within Populations of Four Swedish Hardwood Species Assessed in a Nursery Trial. *Silvae Genetica* 48(1), 17-25.
- BASTIEN C., ALIA R., 2000. What might be useful measures of genetic variability for adaptive traits within populations of Scots pine? *Investigación Agraria: Sistema Recursos Forestales, Fuera de Serie* (1), 97-110.
- BLANCO-SILVA R., FERNÁNDEZ-LÓPEZ J. Application of Knowledge on Genetic variation of Spanish Chestnut Populations to the Selection of Seed Stands Proceeding III International Chestnut Congress, Chaves. *Acta Horticulturae* (under revision).
- BRADSHAW R.H.W., 2004. Past anthropogenic influence on European forests and some possible genetic consequences. *Forest Ecology and Management* 197, 203-212.
- BREISCH H., 1995. Châtaignes et Marrons. Ctifl, 239 pp.
- BUIJTENEN J.P., 1992. Fundamental genetic principles. In: *Handbook of Quantitative Forest Genetics*. Fins L. *et al.*, ed. Kluwer Academic Publisher, pp. 29-68.
- DUCOUSSO A., GUYON J., KREMER A., 1996. Latitudinal and altitudinal variation of bud burst in western populations of sessile oak [*Quercus petraea* (Matt) Liebl]. *Ann Sci For* 53, 775-782.
- ERIKSSON G., EKBERG I., 2001. An Introduction to Forest Genetics. Swedish University of Agricultural Sciences, Uppsala, 166 pp.
- FERNÁNDEZ-LÓPEZ J., ALÍA R., 2003. Technical Guidelines for Genetic Conservation and use for Chestnut (*Castanea sativa*). International Plant Genetic Resources Institute, Rome, Italy. 6 pp.
- FERNÁNDEZ-LÓPEZ J., ARAVANOPoulos F.A., BORTA R., VILLANI F., ALIZOTI P.G., CHERUBINI M., DÍAZ R., MELLANO M.G., ZAS R., ERIKSSON G. Geographic Variability among Extreme European Wild Chestnut Populations, Proceedings III International Chestnut Congress, Chaves. *Acta Horticulturae* (under revision).
- FINESCHI S., TAURCHINI D., VILLANI F., VENDRAMIN G., 2000. Chloroplast DNA polymorphism little geographical structure in *Castanea sativa* Mill. (*Fagaceae*) throughout southern European countries. *Molecular Ecology* 9, 1495-1503.
- GARCÍA-ANTÓN M., MORLA-JUARISTI C., SAINZ-OLLERO H., 1990. Consideraciones sobre la presencia de algunos vegetales relictos terciarios durante el Cuaternario en la Península Ibérica. *Bol R Soc Esp Hist Nat* 86(1- 4), 95-105.
- HÜHN M., 1979. Beiträge zur Erfassung der phänotypischen stabilität. I. Vorschlag einiger auf ranginformationen beruhenden stabilitätsparameter. *EDV in Medizin und Biologie* 11, 112-117.
- JERMSTAD K.D., BASSONI D.L., JECH K.S., WHEELER N.C., NEALE D.B., 2001. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. I. Timing of vegetative bud flush. *Theor Appl Genet* 102, 1142-1151.
- KREMER A., ZANETTO A., DUCOUSSO A., 1997. Multilocus and Multitrait Measures of Differentiation for Gene Markers and Phenotypic Traits. *Genetics* 145, 1229-1241.
- MANINO A., PATETTA A., MARLETTO F., 1991. Investigations on chestnut pollination. 6 th Pollination Symp., *Acta Horticulturae*, ISHS, pp. 335-339.
- MORGENSTERN E.K., 1996. Geographic variation in Forest Trees. University of British Columbia, 209 pp.
- OLIVEIRA D., GOMES A., ILHARCO F., MANTEIGAS A., PINTO J., RAMALHO J., 2001. Importance of Insect

- Pollinators for the Production in the Chestnut, *Castanea sativa*. 8th Pollination Symp., ISHS Acta Horticulturae, pp. 269-273.
- PLIURA A., ERIKSSON G., 2002. Genetic variation in juvenile height and biomass of open pollinated families of six *Castanea sativa* Mill. populations in a  $2 \times 2$  factorial temperature  $\times$  watering experiment. *Silvae Genetica* 51(4), 152-160.
- SAS-INSTITUTE, 1999. SAS/STAT User's guide, version 8. SAS Institute Inc., Cary, NC., 3848.
- SOLIGNAT G., CHAPA J., 1975. Biologie florale. In: Chataignes et marrons. ed. INVUFLEC, Brive, pp. 148.
- SPITZE K., 1993. Population Structure in *Daphnia obtusa*: Quantitative Genetic and Allozymic Variation. *Genetics Society of America* 135, 367-374.
- STERN K., ROCHE L., 1974. Genetics of forest ecosystems. Springer Verlag, 330 pp.
- VILLANI F., PIGLIUCCI M., CHERUBINI M., 1994. Evolution of *Castanea sativa* Mill. in Turkey and Europe. *Genet Res Camb* 63, 109-116.