

Short communication. Inheritance of cotyledon storage proteins in European sweet chestnut (*Castanea sativa* Miller)

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Abstract

A first approximation to the inheritance of cotyledon storage proteins was studied in European sweet chestnut (*Castanea sativa* Mill.) by evaluating the offspring of a controlled cross between two local chestnut varieties (*Corriente* and *Pilonga*) from southern Spain. The analysis was carried out in 15 polymorphic bands corresponding to the albumin fraction of the storage proteins. The relationship between bands displayed one case of allelism and four of linkage. These results should be considered as the baseline of the genetics of these proteins and suggest that they could be useful for the evaluation of the genetic variability in chestnut.

Key words: controlled cross; polymorphism; SDS-PAGE.

Resumen

Comunicación corta. Genética de las proteínas de reserva del cotiledón en castaño (*Castanea sativa* Miller)

Se ha efectuado una primera aproximación a la genética de las proteínas de reserva del cotiledón en castaño (*Castanea sativa* Mill.). Para ello, se ha evaluado la progenie resultante del cruzamiento controlado entre dos variedades locales del sur de España (*Corriente* y *Pilonga*). El análisis se ha realizado en 15 bandas polimórficas correspondientes a la fracción albúmina de las proteínas de reserva. El estudio de la combinación entre bandas ha permitido identificar un caso de alélismo y cuatro de ligamiento. Estos resultados suponen una contribución al estudio de la genética de estas proteínas y sugieren que podrían ser una herramienta útil para la evaluación de la variabilidad genética del castaño.

Palabras clave: cruzamientos controlados; polimorfismo; SDS-PAGE.

European sweet chestnut (*Castanea sativa* Mill.) is the only native species of this genus in Europe and one of the most economically important multipurpose tree species in the Mediterranean region. The species is monoecious, with spatially-separated female and male flowers, and its pollination has been described as anemophilous, entomophilous, or a combination of both (Crane and Walker, 1984). Although there is some information on crosses between *C. sativa* trees (Casasoli *et al.*, 2001), most of the studies on chestnut genetic structure and its reproductive system have been carried out with material from open pollination.

Previous studies have been mainly performed with isozymes (Gillet and Hattemer, 1989; Fineschi *et al.*,

1990; Gillet and Gregorious, 1992). However, other proteins as the storage proteins, present in all plant species seeds, have proved to be useful in evaluation of genetic variability in many species (Gepts, 1990). These proteins can be stored in different tissues (endosperm or cotyledon) and are clearly important in seedlings. The main advantages of these proteins as markers are their high polymorphism level, simple genetic control, environmental independency, as well as the relative speed, ease and low cost of their analysis.

In chestnut cotyledon, the main storage fractions are albumins and globulins. A study on globulins in 20 populations of the species in southern Spain has

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Abbreviations used: SDS (sodium dodecyl sulfate), SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

demonstrated the possibility of the application of this technique to evaluate genetic variability (Alvarez *et al.*, 2003). Likewise, in a posterior study carried out in chestnut, albumins have shown better results than globulins both for the level of polymorphism and for the clear delimitation and identification of bands (Martín *et al.*, 2005). To date, the inheritance of these proteins has not been studied, which could be very useful for future genetic analysis in the species, linking them to the map developed for the species. The main problem for the genetic analysis of variation in cotyledon storage proteins is the necessity of using the offspring from controlled crosses, because the chestnut cotyledon is a diploid tissue in which the male parent is uncertain, due to the reproduction system of this species (alogamy).

This survey describes the variation pattern of cotyledon storage proteins in a controlled cross between two local chestnut varieties (*Corriente* and *Pilonga*) from southern Spain (Martín *et al.*, 2009). These varieties were selected because displayed important differences in morphological traits and genetic profiles and no variation in the flowering time, which facilitated the crosses. The method described by Gallastegui (1926) for obtaining interspecific hybrids resistant to the ink disease (*Phytophthora* sp.) between *C. sativa* × *C. crenata* Siebold & Zucc. was used to perform this controlled cross where both varieties were indistinctly used as male and female. A F₁ full-sib family of 464 offspring was obtained and the inheritance of proteins was evaluated.

A sample of cotyledon (≈100 mg) of each nut was crushed in one mortar and transferred to a 1.5mL-microfuge vial. Cotyledon proteins were sequentially extracted according to the method described by Fonseca *et al.* (1997). Although the four fractions (albumins, globulins, prolamins and glutelins) were obtained, the analysis was only carried out with albumins, according to Martín *et al.* (2005). Albumins were solubilized in buffer [625 mM Tris-HCl pH: 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 2% (w/v) dithiothreitol in a 1:5 ratio (w/v)] and fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at 12% polyacrylamide concentration (w/v, C: 2.67%). The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel at 18°C for 45 min after the tracking dye (bromophenol blue) migrated off the gel. The gels were

stained overnight with 12% (w/v) trichloroacetic acid solution containing 5% (v/v) ethanol and 0.05% (w/v) Coomassie Brilliant Blue R-250. De-staining was carried out with tap water.

The relative mobility (*R_f*) of each band was calculated according to the relationship between the migration distance of one band (cm) and migration distance of the last band (cm); for reference, the first marker was set as 0 and the last band as 1. The segregation of each band (presence vs. absence) in the offspring was evaluated by a Chi-square goodness-of-fit test. The possible cases of relations between bands were calculated according to Ritter *et al.* (1990).

Using a similar methodology as described by Alvarez *et al.* (2003), five zones (named zone A-E) were determined in the SDS-PAGE gel based on the range of molecular weights. Zone A contained the bands with a molecular weight above 67 kDa, zone B between 45 and 67 kDa, zone C between 30 and 45 kDa, zone C between 20 and 30 kDa, and zone E below 14kDa. Each identified band was named with a capital letter for the gel zone followed by a number corresponding to the relative mobility (Table 1).

In all of the material analysed from the controlled cross, 52 bands were identified, 32 of which were polymorphic (Table 1). Zones C and D showed the highest number of bands with 13 and 14 bands, respectively, whereas only three bands were found in the zone A. The zones that showed the highest levels of polymorphism were zones C, D and E, in which 24 of 32 polymorphic bands appeared (7, 11 and 6, respectively). However, only 15 out of the 32 bands displayed the required resolution to be monitored in the offspring. These 15 bands appear in italics in Table 1.

According to the presence (P) or absence (A) of each band, the progenitors could be PP, PA or AA for each band. Furthermore, the genetics of a band can only be studied if both progenitors are heterozygote for the band (PA / PA) or if one progenitor is heterozygote and the other homozygote for the absence of the band (PA / AA). In the first case, the expected segregation would be 3:1 and 1:1 in the second. In the offspring, twelve bands were adjusted to 3:1 segregation and three presented 1:1 segregation (Table 2).

The possible combinations of pairs of bands showed a case of allelism between E84 and E85, given that all individuals displayed either one or the other of the two bands, but no simultaneous presence or absence was detected. In the remaining relationships among pairs

Table 1. Characterisation according to the relative mobility (Rf) of the 52 bands identified in the offspring of the controlled cross

Bands	Rf ± s.d.	M/P ^a	Bands	Rf ± s.d.	M/P ^a
97 kDa	0	–	D51	0.511 ± 0.009	P
A6	0.056 ± 0.005	M	D53	0.532 ± 0.003	P
A7	0.070 ± 0.005	P	D54	0.543 ± 0.007	P
A8	0.081 ± 0.004	P	D56	0.556 ± 0.007	M
66 kDa	0.080 ± 0.005	–	D57	0.572 ± 0.008	P
B9	0.090 ± 0.002	M	D59	0.592 ± 0.010	P
B10	0.100 ± 0.003	P	D62	0.616 ± 0.010	P
B11	0.115 ± 0.003	P	D63	0.627 ± 0.005	M
B13	0.132 ± 0.004	M	D65	0.653 ± 0.010	P
B14	0.141 ± 0.004	M	D66	0.659 ± 0.009	P
B15	0.153 ± 0.003	M	D67	0.672 ± 0.008	P
45 kDa	0.228 ± 0.004	–	20 kDa	0.702 ± 0.004	–
C22	0.223 ± 0.003	M	E70	0.701 ± 0.004	M
C23	0.233 ± 0.009	P	E72	0.719 ± 0.010	P
C25	0.250 ± 0.002	M	E73	0.732 ± 0.008	P
C26	0.266 ± 0.005	P	E76	0.763 ± 0.003	M
C28	0.279 ± 0.005	M	E78	0.779 ± 0.002	P
C30	0.297 ± 0.003	M	E80	0.802 ± 0.001	M
C31	0.312 ± 0.003	M	E82	0.822 ± 0.001	M
C33	0.334 ± 0.004	P	E84	0.838 ± 0.003	P
C35	0.349 ± 0.004	P	E85	0.846 ± 0.003	P
C38	0.378 ± 0.002	P	E86	0.862 ± 0.002	P
C39	0.393 ± 0.002	M	14 kDa	0.904 ± 0.004	–
C41	0.409 ± 0.005	P	F89	0.892 ± 0.005	P
C45	0.448 ± 0.006	P	F92	0.920 ± 0.003	M
30 kDa	0.458 ± 0.005	–	F94	0.941 ± 0.001	P
D47	0.471 ± 0.005	P	F95	0.953 ± 0.002	P
D48	0.482 ± 0.005	M	F98	0.979 ± 0.001	M
D50	0.499 ± 0.008	P	F100	1	P

s.d. = standard deviation. ^a M = monomorphic band; P = polymorphic band.

Table 2. Polymorphic bands studied in the offspring. Presence/Absence cases and adjusts to 1:1 or 3:1 segregation

Bands	Presence	Absence	χ^2 (1:1)	χ^2 (3:1)
D47	279	104	79.96***	0.95ns
D50	250	106	58.25***	4.32*
D51	194	157	3.90ns	72.87***
D53	121	93	3.34 ns	38.88***
D54	87	89	0.02 ns	61.36***
E72	92	27	35.50**	0.17 ns
E73	94	95	0.01 ns	64.35***
E78	75	79	0.10 ns	56.80***
E84	116	123	0.21 ns	89.27***
E85	119	119	0.00 ns	79.3***
E86	93	78	1.32 ns	38.75***
F89	54	52	0.00 ns	32.72***
F94	134	125	0.31 ns	74.74***
F95	139	121	1.25 ns	64.32***
F100	139	130	0.30 ns	78.07***

ns = non significant; * p < 0.05; ** p < 0.01; and *** p < 0.001.

of bands, 63 cases adjusted to the expected proportion of independence: 26 cases were adjusted to 1:1:1:1 segregation (both progenitors segregated according to a 1:1 ratio), 34 cases to 3:3:1:1 (one progenitor 3:1 and the other 1:1), and three cases to 9:3:3:1 (both progenitors were 3:1). Furthermore, four linkage cases were detected between bands (D47/D51, D50/D51, E73/E78 and F94/F95) with recombination distances between 26 and 38 cM (Table 3).

In conclusion, current data suggest that these proteins could be a useful tool for the evaluation of the genetic variability in chestnut. The main advantages are the ease and speed of the procedure, along with its relative low cost. Nevertheless, due to the diploid nature of the studied tissue and the complex nature of the protein profile, its evaluation requires staff with considerable experience. In addition, the inability to automate of the analysis could be another important handicap.

Table 3. Segregation data and chi-square test of linkage analysis

Pair	PP	PA	AP	AA	χ^2 (3:3:1:1)	χ^2 (1:1:1:1)	Distance (cM)
D47/D51	139	94	32	49	12.44**	–	25.80
D50/D51	123	127	72	33	21.59***	–	29.58
E73/E78	28	15	11	33	–	14.86**	29.89
F94/F95	53	76	78	42	–	15.05**	38.15

** p < 0.01; and *** p < 0.001.

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