JOURNAL OF FOREST SCIENCE, *55*, 2009 (11): 526–531

SHORT COMMUNICATION

The use of cotyledon proteins to assess the genetic diversity in sweet holm oak

M. A. Martín[1], R. Navarro-Cerrillo[2], P. Ortega[1,2], J. B. Alvarez[1]

[1]*Departamento de Genética, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Córdoba, Spain*
[2]*Departamento de Ingeniería Agroforestal, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Córdoba, Spain*

ABSTRACT: Sweet holm oak (*Quercus ilex* ssp. *ballota* Desf. Samp.) is an important broad-leaved tree spread in the Mediterranean basin. In Spain, few studies on the genetic variability of this species have been displayed. Storage seed proteins are a useful tool in the evaluation of the genetic variability of many species. The objective of this study was to analyze the usefulness of cotyledon proteins as markers of the genetic diversity in sweet holm oak. The evaluated populations were highly polymorphic for the glutelins, being detected up to 32 polymorphic bands with a wide distribution among all them. Considering all evaluated populations, about 35.8% of the total allelic variation was distributed among populations. This method of analysis of cotyledon storage proteins (glutelins) could be considered an additional tool for the evaluation of genetic diversity in this species.

Keywords: seed storage proteins; genetic resources; sweet holm oak

Storage seed proteins have proved to be a useful tool to evaluate genetic variability in many species (Gepts 1990), and have been used as an important genetic marker in some species, mainly in cereals in which their variability is related to technological properties of the flour (Wrigley et al. 2006). The main advantages of these proteins as markers are the high polymorphism level, simple genetic control, environmental independence, and the economy, easiness and expeditiousness of their analysis. Although the role of these proteins in forest species has been scarcely studied, a few works have been carried out on Fagaceae, mainly on their biochemical characteristics (Collada et al. 1986, 1991; Fonseca et al. 1997) and on their genetic diversity (Alvarez et al. 2003; Martín et al. 2005).

Holm oak (*Quercus ilex* L.) is a wide-spread broad-leaved tree species in the Mediterranean basin. In Spain, it occupies 2,039,563 ha and the main stands are found in the south and west (Jiménez et al. 1996). In southern Spain (Andalusia), with 735,671 ha, this species is associated with the *dehesa* system, which is of great value for agriculture, livestock and forestry. These zones were included in the Natura 2000 Network for the European Union for landscape and environmental importance.

In the Iberian Peninsula, two main subspecies were found: ssp. *ilex* and ssp. *ballota* (Desf.) Samp. The main differences between both subspecies are the leaf morphology and pubescence, the number of secondary nerves, and leaf size (Castroviejo et al. 1990). The acorn taste is also different; the ssp. *ilex* is mainly bitter

Table 1. Frequencies of each band in 120 acorns and 8 populations of holm oak

Zone	Band	Acorn (n = 120)		Population (n = 8)	
		N	(%)	N	(%)
C	1C	99	82.5	8	100.0
	2C	78	65.0	8	100.0
	3C	111	92.5	8	100.0
	4C	118	98.3	8	100.0
	5C	116	96.7	8	100.0
	6C	10	8.3	4	50.0
	7C	53	44.2	6	75.0
	8C	60	50.0	8	100.0
	9C	20	16.7	5	62.5
	10C	32	26.7	7	87.5
	11C	15	12.5	6	75.0
	12C	45	37.5	7	87.5
	13C	29	24.2	8	100.0
	14C	2	1.7	1	12.5
	15C	67	55.8	8	100.0
D	1D	56	46.7	7	87.5
	2D	32	26.7	7	87.5
	3D	31	25.8	7	87.5
	4D	10	8.3	4	50.0
	5D	33	27.5	7	87.5
	6D	98	81.7	8	100.0
	7D	31	25.8	7	87.5
	8D	86	71.7	7	87.5
	9D	26	21.7	8	100.0
	10D	15	12.5	6	75.0
	11D	115	95.8	8	100.0
E	1E	55	45.8	6	75.0
	2E	65	54.2	6	75.0
	3E	100	83.3	8	100.0
	4E	74	61.7	8	100.0
	5E	87	72.5	8	100.0
	6E	83	69.2	8	100.0

while the ssp. *ballota* is sweet, so that it is commonly known as sweet holm oak. Up to seven botanical varieties have been identified in the ssp. *ballota*: var. *avellanaeformis*, var. *brevicupulata*, var *crassicupulata*, var. *dolichocalyx*, var. *expansa*, var. *macrocarpa* and var. *rotundifolia* (Vázquez-Pardo 1998).

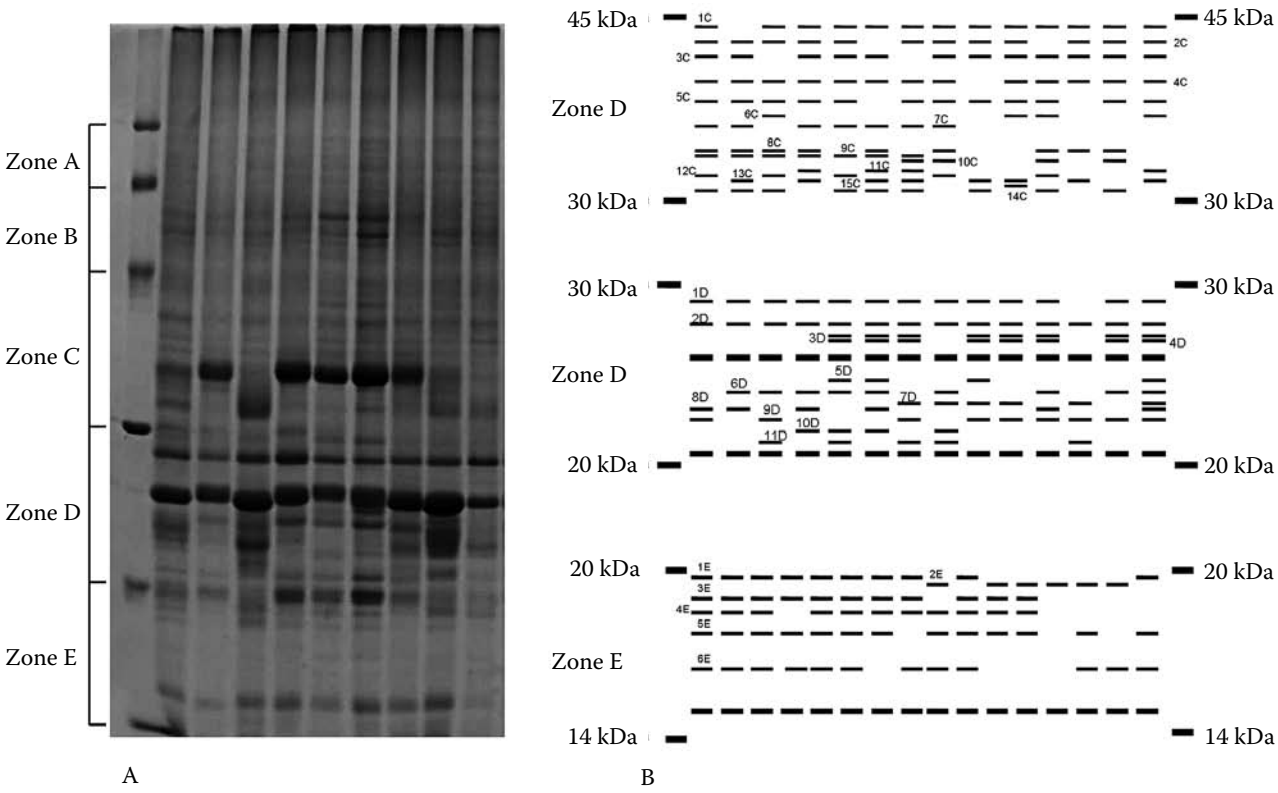

Fig. 1A. SDS-PAGE of glutelins from cotyledons of holm oak

Fig. 1B. Diagrammatic representation of each zone evaluated, zone C (upper), zone D (medium) and zone E (lower)

The aim of the present study was to evaluate cotyledon storage proteins as markers of the genetic diversity in sweet holm oak.

MATERIAL AND METHODS

Samples of acorns from 40 holm oak trees collected from the principal distribution regions of this species in Andalusia (south of Spain) were used. These materials were grouped in eight populations with five trees per population, four for Cordoba province (CO-1 to CO-4) and four for Seville province (SE-01 to SE-04). Three acorns per tree were analyzed.

Previous to protein extraction, the samples (≈ 50 mg of cotyledon) were un-lipped with diethyl ether and acetone. Cotyledon proteins were sequentially extracted according to the method described by Fonseca et al. (1997). Four fractions (albumins, globulins, prolamines and glutelins) were obtained, all of them were precipitated with 1 ml of cold acetone, and the dried pellets were solubilized in buffer containing 625mM Tris-HCl pH: 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 2% (w/v) dithiothreitol at a ratio 1:5 (w/v).

The electrophoretic analyses were carried out in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at a 10% or 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 30 min after the tracking dye migrated off the gel. 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. Destaining was carried out with tap water.

The expected heterozygosity (H_e) was calculated in all populations. The genetic diversity over all populations (H_t) together with the average genetic diversities within (H_s) and among (D_{st}) populations were calculated according to Nei (1973). The relative magnitude of genetic differentiation among populations, G_{st}, was estimated as D_{st}/H_t.

RESULTS AND DISCUSSION

Of the four fractions analyzed, glutelins showed the best results with up to 32 polymorphic bands. Five zones were established in the gel by the molecular weight range named as zones A–E (Fig. 1A). The polymorphic bands were distributed in zones C, D and E (Fig. 1A). Fifteen, eleven and six bands were detected in each zone, respectively (Fig. 1B).

Table 2. Differentiation of globulin diversity within and among eight populations of holm oak

Population	*N* acorns	H_t	H_s	D_{st}	G_{st}
CO-01	15	0.156	0.067	0.089	57.1
CO-02	15	0.229	0.200	0.029	12.5
CO-03	15	0.201	0.125	0.076	37.7
CO-04	15	0.277	0.150	0.127	45.8
Cordoba	*60*	*0.328*	*0.214*	*0.114*	*34.8*
SE-01	15	0.234	0.156	0.079	33.6
SE-02	15	0.159	0.108	0.051	31.8
SE-03	15	0.187	0.106	0.082	43.6
SE-04	15	0.247	0.158	0.088	35.8
Seville	*60*	*0.283*	*0.207*	*0.077*	*27.0*
Overall	***120***	***0.328***	***0.211***	***0.117***	***35.8***

H_t – total gene diversity, H_s – average gene diversity within populations, D_{st} – average gene diversity among populations, G_{st} – gene diversity among populations relative to H_t

The frequencies of each band are shown in Table 1.

The classification of Marshall and Brown (1975) was used to assess the distribution of alleles in different populations. In general, the bands presented a wide distribution among all populations. The bands that showed a low frequency appeared in two types of distribution: the band 14C that only appeared in Seville population (SE-01) can be considered rare (frequency ≤ 5%), the band 6C, although with low frequency (8.3%), appeared in the four populations from Seville, and the other low frequent band (4D) was detected in one population from Cordoba (CO-03) and three from Seville (SE-01, SE-02 and SE-04) (Table 1). According to this classification, the first two bands may be considered of local distribution and the third of wide distribution.

The highest polymorphic populations were CO-02 and SE-01, which presented variation in 26 bands. The expected heterozygosity (H_e) showed a mean value of 0.211, ranging from 0.156 in population CO-03 to 0.277 in population CO-02. Thus, the value of H_e in our study was similar to the value (H_e = 0.214 or H_e = 0.227) in the other Fagaceae species (Danne et al. 1999; Alvarez et al. 2003).

The characterization of the diversity in holm oak for glutelin proteins is present in Table 2. The genetic diversity ranged between H_t = 0.156 for population CO-01 and H_t = 0.277 for population CO-04. The genetic diversity found in populations from Cordoba (H_t = 0.328) was equal to the total genetic diversity (H_t = 0.328), while populations from Seville showed a lower value (H_t = 0.283). The 27.0% of the genetic diversity of this last group was detected among populations; however, this value was higher in Cordoba with 34.8% of total genetic diversity. The proportion of genetic diversity found among the holm oak populations evaluated (G_{st} = 35.8%) was similar to the data obtained in other Fagaceae such as sweet chestnut using the same marker (*C. sativa*, G_{st} = 39.3%, Alvarez et al. 2003) and somewhat higher than that observed with isozymes in the same species (F_{st} = 10.0%; Villani et al. 1991) or other species of the genus (*C. dentata*, G_{st} = 11.0%; Huang et al. 1998). However, because the diversity was measured with different genetic markers from those applied in our work, this could affect the level of genetic diversity detected.

When the trees evaluated were classified according to botanical varieties, thirty-seven out of forty could be associated with three botanical varieties (var. *crassicupulata*, var. *macrocarpa* and var. *rotundifolia*). The main variety was var. *rotundifolia* with twenty trees, while var. *crassicupulata* was represented by three trees only. The var. *macrocarpa* was separated into two groups according to acorn weight; trees with small acorns (9) were included in the var. *microcarpa* and trees with large ones (5) were classified as var. *macrocarpa* in a narrow sense, which appeared only in Seville populations.

The materials used in the present work were collected in some representative regions of holm oak

distribution in Andalusia. Although all protein fractions were analyzed, the best results were obtained with the glutelin fraction, which showed a high degree of polymorphism, finding up to 32 polymorphic bands in all the trees evaluated. On the other hand, the understanding of the genetic diversity presents in a species and the distribution of this variation among populations is important to set up appropriate management strategies, mainly in reforestation. In this respect, this method of analyzing cotyledon storage proteins (glutelins) could be considered an additional tool to shed light on the evaluation of genetic diversity in this species.

Acknowledgements

The first author is grateful to the Alfonso Martín Escudero Foundation for a postdoctoral fellowship.

References

ALVAREZ J.B., MUÑOZ-DIEZ C., MARTÍN-CUEVAS A., LÓPEZ S., MARTÍN L.M., 2003. Cotyledon storage proteins as markers of the genetic diversity in *Castanea sativa* Miller. Theoretical and Applied Genetics, *107*: 730–735.

CASTROVIEJO S., LAÍNZ M., LÓPEZ G., MONSERRAT P., MUÑOZ F., PAIVA J., VILLAR L., 1990. Flora Ibérica. Plantas vasculares de la Península Ibérica e Islas Baleares. Vol. 2. C.S.I.C. Madrid, Real Jardín Botánico.

COLLADA C., CASADO R., BARBER D., FERNANDEZ DE CALEYA R., ARAGONCILLO C., 1986. Characterization of seed protein fractions from *Castanea* spp. Journal of Experimental Botany, *37*: 1872–1878.

COLLADA C., CABALLERO R.G., CASADO R., ARAGONCILLO C., 1991. Seed storage proteins in Fagaceae: similarity between *Castanea* globulins and *Quercus* glutelins. Plant Science, *75*: 145–154.

DANNE F., HAWKINS L.K., HUANG H., 1999. Genetic variation and population structure of *Castanea pumila* var. *ozarkensis*. Journal of the American Society for Horticultural Science, *124*: 666–670.

FONSECA P.A., FERREIRA R.B., TEIXEIRA A.R., 1997. Seed proteins from *Quercus suber*. Journal of Agriculture and Food Chemistry, *45*: 3443–3447.

GEPTS P., 1990. Genetic diversity of seed storage proteins in plants. In: BROWN A.H.D., CLEGG M.T., KAHLER A.L., WEIR B.S. (eds), Plant Population Genetics, Breeding and Genetic Resources. Suderland, Sinauer Associates Inc. Publishers: 64–82.

HUANG H., DANNE F., KUBISIAK T.L., 1998. Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut *Castanea dentata* (Fagaceae). American Journal of Botany, *85*: 1013–1021.

JIMÉNEZ M.P., DÍAZ-FERNÁNDEZ P.M., IGLESIAS S., DE TUERO M., GIL L., 1996. Las regiones de procedencia de *Quercus ilex* L. en Espańa. Madrid, ICONA.

LAEMMLI U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, *227*: 680–685.

MARSHALL D.R., BROWN A.H.D., 1975. Optimum sampling strategies in genetic conservation. In: FRANKEL O.H., HAWKES J.G. (eds), Crop Genetic Resources for Today and Tomorrow. Cambridge, Cambridge University Press: 53–70.

MARTÍN M.A., MARTÍN L.M., ALVAREZ J.B., 2005. Cotyledon storage proteins in European sweet chestnut. Acta Horticulturae, *693*: 459–463.

NEI M., 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences, USA, *70*: 3321–3323.

VÁZQUEZ PARDO F.M., 1998. Semillas del género *Quercus* L. (Biología ecología y manejo). Consejería de Agricultura y Comercio. Junta de Extremadura, Badajoz.

VILLANI F., PIGLIUCCI M., BENEDETTELLI S., CHERUBINI M., 1991. Genetic differentiation among Turkish chestnut (*Castanea sativa* Mill.) populations. Heredity, *66*: 131–136.

WRIGLEY C., BEKES F., BUSHUK W. (eds), 2006. Gliadin and Glutenin: the Unique Balance of Wheat Quality. St. Paul, AACC International Press.

Received for publication October 15, 2008
Accepted after corrections May 11, 2009

Použití proteinů kotyledonu k hodnocení genetické diverzity dubu cesmínového okrouhlolistého

ABSTRAKT: Dub cesmínový okrouhlolistý (*Quercus ilex* ssp. *ballota* Desf. Samp.) je důležitým listnatým stromem rozšířeným ve středozemní oblasti. Ve Španělsku bylo publikováno několik studií o genetické variabilitě tohoto druhu. Zásobní proteiny semen jsou užitečným nástrojem při hodnocení genetické variability mnoha druhů. Cílem práce

bylo analyzovat proteiny kotyledonů a jejich využití jako ukazatele genetické diverzity dubu cesmínového okrouhlolistého. Hodnocené populace byly vysoce polymorfní z hlediska glutelinu, mezi všemi zkoumanými populacemi bylo detekováno až 32 polymorfních proužků. Vezmeme-li v úvahu všechny hodnocené populace, okolo 35,8 % z celkové proměnlivosti alel bylo rozděleno mezi populace. Tato metoda analýzy zásobních proteinů kotyledonu (glutelinů) může být použita jako doplňkový nástroj pro hodnocení genetické diverzity tohoto druhu.

Klíčová slova: zásobní proteiny semen; genetické zdroje; dub cesmínový okrouhlolistý

Corresponding author:

Prof. Dr. Juan B. Alvarez, Universidad de Cordoba, Departamento de Genetica, Escuela Tecnica Superior de Ingenieros Agronomos y de Montes, Edificio Gregor Mendel, Campus de Rabanales, ES-14071 Cordoba, Spain
tel.: + 349 5721 8505, fax: + 349 5721 8503, e-mail: jb.alvarez@uco.es