Use of Highly Discriminating Morphological Characters and Isozymes in the Study of Spanish Chestnut Cultivars

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ABSTRACT. Morphological characters (six traits) and isozymes (four systems, five loci) were used to discriminate between Spanish chestnut cultivars (*Castanea sativa* Mill.) from the Iberian Peninsula. A total of 701 accessions (representing 168 local cultivars) were analyzed from collections made between 1989 and 2003 in the main chestnut growing areas: 31 were from Andalucía (12 cultivars), 293 from Asturias (65 cultivars), 25 from Castilla-León (nine cultivars), four from Extremadura (two cultivars) and 348 from Galicia (80 cultivars). Data were synthesized using multivariate analysis, principal component analysis, and cluster analysis. A total of 152 Spanish cultivars were verified: 58 cultivars of major importance and 94 of minor importance, of which 18 had high intracultivar variation. Thirty-seven cultivars were clustered into 14 synonymous groups. Six of these were from Galicia, one from Castilla-León (El Bierzo), four from Asturias, one from Asturias and Castilla-León (El Bierzo), and two from Asturias, Castilla-León (El Bierzo), and Galicia. The chestnut cultivars from Galicia and Asturias were undifferentiated in genetic terms, indicating that they are not genetically isolated. Overall, chestnut cultivars from southern Spain showed the least variation. Many (58%) of Spanish cultivars produced more than 100 nuts/kg; removing this low market-value character will be a high priority. The data obtained will be of use in chestnut breeding programs in Spain and elsewhere.

Growers have selected chestnut cultivars since Roman times. Traditionally, chestnut stands are derived by grafting selected cultivars onto planted seedlings. In the Middle Ages huge plantations were established for forestry with the dual purpose of timber and nut production (Pereira-Lorenzo and Ramos-Cabrer, 2004). Some of these plantations can still be found in Tuscany (Italy), Galicia (Spain), Tras-Os-Montes (Portugal), and Aveyron and Cévennes (France). Monasteries established most of these extensive chestnut orchards.

There are 187 cultivars documented in Spain (Pereira-Lorenzo et al., 2001a, 2001b). There is considerable need to define precisely chestnut cultivar variation for improved planting regimes and chestnut breeding. Breviglieri (1955) made the first comprehensive study of chestnut variation using morphological characteristics in the “Scheda Castanografica.” Later, the International Union for the Protection of New Varieties of Plants (UPOV) published a chestnut guide for distinguishing chestnut cultivars and suggested some of the main discriminant characteristics (UPOV, 1988). Recently, some researchers have applied these guidelines to Spanish cultivars (Pereira-Lorenzo et al., 1996a; Ramos-Cabrer and Pereira-Lorenzo, 2005), Portuguese cultivars (Goulao et al., 2001), and to different chestnut species (Oraguzie et al., 1998).

Isozymes were the first markers used to study the genetic variation between chestnut cultivars (Pereira-Lorenzo et al., 1996b; Ramos-Cabrer and Pereira-Lorenzo, 2005; Sawano et al. 1984) and natural chestnut populations (Aravanopoulos et al., 2002; Pigliucci et al., 1990a, 1990b; Villani et al., 1991a, 1991b). Multivariate analysis has proved useful to discriminate genetic relationships within germplasm of *Castanea sativa* (Pereira-Lorenzo et al., 1996a, 1996b), *C. dentata* Borkh. (Huang et al., 1998; Oraguzie

et al., 1998), and *C. pumila* Mill. (Dane and Hawkins, 1999). A previous study of Spanish chestnuts showed a reduction in genetic variation (Ramos-Cabrer and Pereira-Lorenzo, 2005). However, no current data exist for the most important chestnut growing region, Asturias, in northern Spain. Therefore, the objectives here were to 1) classify Spanish chestnut cultivars for conservation and propagation purposes; and 2) determine the total variation of Spanish chestnuts. The data generated have significant implications for chestnut breeding programs.

Materials and Methods

PLANT MATERIAL. An inventory of some of the grafted cultivars included in this work and their locations has been published previously (Pereira-Lorenzo et al., 2001a). Accessions are a representative sample of cultivars cultivated in Spain. In 1999, a total of 293 accessions corresponding to 65 named cultivars were found in 145 different locations in 49 Councils from Asturias (Fig. 1; Table 1). Some of those cultivars were reported previously by Fernández-Lamuño (1984). Cultivars located in Asturias were compared with 348 accessions from 80 cultivars collected in Galicia from 1989–90, 31 accessions from 12 cultivars collected in Andalucía from 1996–2001, 25 accessions from nine cultivars collected in Castilla-León from 1996–2001, and four accessions from two cultivars collected in Extremadura from 1996–2001 (Pereira-Lorenzo et al., 1996a, 1996b, 2001a; Ramos-Cabrer and Pereira-Lorenzo, 2005). From each tree, nuts with burs were collected for 2 years, or more often, 3 years. Samples of catkins were also collected and preserved for further study.

MORPHOLOGY AND PHENOLOGY. Traits were selected using UPOV (1988) criteria, some of which had previously been used to classify cultivars from Galicia (Pereira-Lorenzo et al., 1996a), Andalucía, Castilla-León, and Extremadura (Ramos-Cabrer and Pereira-Lorenzo, 2005). Three traits were quantitative: 1) nut

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Fig. 1. Spanish chestnut areas where 168 cultivars were localized and 152 classified.

size expressed as number/kilogram (NNK) and weight of lateral nuts in grams (WLN), 2) nut width to length ratio ($WLR = 100 \times \text{width/length}$), and 3) percentage of divided nuts (PDN). Three traits were qualitative: 1) type of catkin: astaminate (CAS), brachystaminate (CBR), mesostaminate (CME), and longistaminate (CLO); 2) size of bur spines: short (BTS), medium (BTM) and long (BTL); and 3) harvesting time: very early [VEA (15–25 Sept.)], early [EAR (26 Sept.–10 Oct.)], medium [MED (11–25 Oct.)], late [LAT (26 Oct.–11 Nov.)], and very late [VEL (after 11 Nov.)]. A new binomial variable was created for each class of qualitative trait, where the values 1 or 0 indicate presence or absence of the observed quality in the individual phenotype recorded (Kaufman and Rosseeuw, 1990). Thus, the three qualitative traits were split into 12 binomial variables.

ISOZYMES. Four isozyme systems were used (Pereira-Lorenzo, 1996b): isocitrate dehydrogenase (IDH; EC 1.1.1.42), phosphoglucose isomerase (PGI; EC 5.3.1.9), malate dehydrogenase (MDH; EC 1.1.1.37), and shikimate dehydrogenase (SKDH; EC 1.1.1.25). An isotype was considered rare when its frequency was lower than 0.05.

ANALYSIS. To quantify genetic variation, data were analyzed using Fstat version 2.93 (Goudet, 1995) and Genepop version 3.3 (Raymond and Rousset, 1995) using the methods of Weir and Cockerham (1984) to estimate F statistics: the inbreeding coefficient (F_{is}), the total fixation index (F_{it}), and the proportion of differentiation among the populations (F_{st}) (Wright, 1951).

Morphology variation was expressed as an average value for quantitative traits and as a frequency for qualitative traits. The relationships among accessions, using the 12 morphological traits and 13 isotypes, were examined with a principal components analysis (PCA) on the correlation matrix of the accession means [PRINCOMP of SAS (release 6.03; SAS Institute Inc., Cary, N.C.)]. The Mahalanobis generalized distance (D^2 of Mahalanobis) (Goodman, 1972, 1973) was calculated as a dissimilarity

measure between accessions from the standardized principal components (PCs) that had eigen values higher than one ($\lambda_k > 1$). The distance matrix was used in a cluster analysis (UPGMA) (Sneath and Sokal, 1973) to classify accessions and cultivars by their genetic similarity.

Results

ISOZYMES. Frequencies of isotypes for Asturian chestnut cultivars were similar to those previously found in Galicia (Pereira-Lorenzo et al., 1996a), except that there were fewer Mdh3-a and Idh-b isotypes. Idh-b was a rare isotype in Asturias and its frequency was closer to that found in middle and southern Spain (Castilla-León, Extremadura, and Andalucía) (Table 2). Isotype frequencies in Galicia are generally representative of those in the whole of Spain. Only Pgi-b can be considered a rare isotype in Galicia, Castilla-León, and Asturias, and is completely absent in Andalucía and Extremadura. The lower frequency of Idh-b found in Asturias was the main difference between Asturias and Galicia (representative of Spanish chestnuts in general). As reported previously (Pereira-Lorenzo et al., 1996b; Ramos-Cabrer and Pereira-Lorenzo, 2005), the isotype frequencies of cultivars were different from those of the wild populations studied in France (Bonnetoi, 1984), Italy (Villani et al., 1991a), and Turkey (Villani et al., 1991b). The observed heterozygosity (H_o) was higher than the expected heterozygosity (H_e) as was found previously (Pereira-Lorenzo et al., 1996b). The inbreeding coefficient (F_{is}) in Galicia showed a significant ($P < 0.05$) departure from Hardy-Weinberg proportions, with an excess of heterozygotes at each locus. In contrast, Mdh3 and Pgi loci in Asturias did not show significantly departure from Hardy-Weinberg expectations. The average multilocus inbreeding coefficient (F_{is}) for all samples was -0.132 , a significant ($P < 0.001$) overall departure from Hardy-Weinberg proportions.

Table 1. Chestnut accessions and cultivars from five regions of Spain studied by morphology and isoenzymes.

Accessions (no.)	Cultivar	Region	Accessions (no.)	Cultivar	Region	Accessions (no.)	Cultivar	Region
1	Capilla	Andalucía	7	Pelona	Asturias	2	Galega	Galicia
2	Comisaria	Andalucía	2	Pelosa	Asturias	7	Garrida	Galicia
2	Corriente	Andalucía	4	Ramiega	Asturias	1	Garriga	Galicia
2	Dieguina	Andalucía	15	Rapuca	Asturias	2	Horrea	Galicia
2	Helechal	Andalucía	1	Ravexa	Asturias	10	Inxerta	Galicia
4	Pelona	Andalucía	3	Roxa	Asturias	3	Lebre	Galicia
6	Pilonga	Andalucía	1	Roxina	Asturias	4	Lemés	Galicia
4	Planta Alajar	Andalucía	2	Rubia	Asturias	8	Longal	Galicia
1	Rubia	Andalucía	1	Seronda	Asturias	12	Loura	Galicia
3	Temprana	Andalucía	4	Sevillana	Asturias	11	Luguesa	Galicia
2	Tomasa	Andalucía	1	Tamón	Asturias	1	Mazaño	Galicia
2	Vázquez	Andalucía	1	Tixera	Asturias	1	Migueliña	Galicia
2	Argua	Asturias	29	Valduna	Asturias	1	Monfortina	Galicia
2	Baragaña	Asturias	8	Vaquera	Asturias	13	Negral	Galicia
1	Boroña	Asturias	4	Vegamesada	Asturias	3	Ouriza	Galicia
1	Brullina	Asturias	15	Verde	Asturias	2	Outeira	Galicia
2	Calva	Asturias	2	Villaviciosa	Asturias	2	País	Galicia
1	Cañuela	Asturias	2	Vizcaña	Asturias	1	Pallarega	Galicia
1	Caranquexa	Asturias	2	Xidra	Asturias	1	Paradesa	Galicia
1	Carreña	Asturias	1	Ximara	Asturias	16	Parede	Galicia
1	Cerbana	Asturias	5	Zapatona	Asturias	1	Patacudá	Galicia
15	Chamberga	Asturias	1	Brava	Castilla-León	5	Pelado	Galicia
1	Chancloia	Asturias	2	Gallego	Castilla-León	1	Peluda	Galicia
1	Cofina	Asturias	6	Injerto, Injerta	Castilla-León	2	Piconá	Galicia
1	Colunga	Asturias	3	Marelo	Castilla-León	11	Pontearreas	Galicia
4	Crespa	Asturias	8	Negral	Castilla-León	2	Porteliña	Galicia
1	Cruz	Asturias	2	Parede	Castilla-León	2	Portugués	Galicia
10	Doriga	Asturias	1	Rapado	Castilla-León	4	Pozoredondo	Galicia
1	Escamplero	Asturias	1	Rapega	Castilla-León	4	Praga d'Afora	Galicia
1	Fano	Asturias	1	Verdello	Castilla-León	9	Presa	Galicia
2	Feltrona	Asturias	1	Injerta	Extremadura	1	Puga	Galicia
8	Forniega	Asturias	3	Verata	Extremadura	5	Puga do Bolo	Galicia
14	Galega	Asturias	3	Abadá	Galicia	1	Puga do Receiro	Galicia
5	Grúa	Asturias	1	Abarcá	Galicia	16	Raigona	Galicia
8	Injerta	Asturias	25	Amarelante	Galicia	1	Rañuda	Galicia
3	Largaña	Asturias	1	Anaxa	Galicia	7	Rapada	Galicia
3	Leinova	Asturias	1	Anchas	Galicia	1	Redondo	Galicia
9	Llanisca	Asturias	2	Arial	Galicia	2	Riá	Galicia
1	Loura	Asturias	5	Bermella	Galicia	1	Ribeirá	Galicia
1	Mariana	Asturias	16	Blanca	Galicia	1	Rosenda	Galicia
2	Marimoeche	Asturias	1	Bolesa	Galicia	1	Rozada	Galicia
2	Marina	Asturias	5	Bravoleirado	Galicia	5	Salnesa	Galicia
1	Mediana	Asturias	3	Cabezuda	Galicia	2	Sergude	Galicia
4	Miguelina	Asturias	4	Calva	Galicia	2	Serodia	Galicia
1	Montesín	Asturias	3	Campilla	Galicia	1	Soutogrande	Galicia
2	Mourisco	Asturias	1	Carrelao	Galicia	1	Tarabelao	Galicia
10	Nargana	Asturias	1	Castellana	Galicia	9	Temperá	Galicia
4	Navexa	Asturias	1	Caurelal	Galicia	2	Torbeana	Galicia
3	Negral	Asturias	2	Cedo	Galicia	1	Toubesa	Galicia
7	Ordaliega	Asturias	3	Cerdedo	Galicia	1	Veiguiña	Galicia
4	Padana, Pagana	Asturias	2	Cerreda	Galicia	8	Ventura	Galicia
1	Palaciana	Asturias	7	Courelá	Galicia	17	Verde	Galicia
1	Panchina	Asturias	3	Culona	Galicia	2	Vileta	Galicia
33	Parede	Asturias	1	Curuxa	Galicia	1	Villarenga	Galicia
6	Parruca	Asturias	2	Desgrañadiza	Galicia	7	Xabrega	Galicia
1	Pelgo	Asturias	16	Famosa	Galicia	1	Xilimendra	Galicia
						701	Total	

Table 2. Estimates of genetic diversity parameters in 611 accessions corresponding to 163 chestnut cultivars located in five regions of Spain: Asturias, Galicia, and Castilla-León in northern Spain; Extremadura in central Spain; and Andalucía in southern Spain.

		Allele frequencies					
Enzyme	Allele	Spain N = 611 ^z	Asturias N = 258	Galicia N = 300	Andalucía N = 28	Castilla-León N = 21	Extremadura N = 4
Mdh1	<i>Mdh1-b</i>	1.000	1.000	1.000	1.000	1.000	1.000
Mdh2	<i>Mdh2-a</i>	0.736	0.653	0.807	0.768	0.762	0.500
	<i>Mdh2-b</i>	0.264	0.347	0.193	0.232	0.238	0.500
Mdh3	<i>Mdh3-a</i>	0.159	0.091	0.217	0.125	0.238	0.000
	<i>Mdh3-b</i>	0.841	0.909	0.783	0.875	0.762	1.000
Skdh	<i>Skdh-a</i>	0.420	0.432	0.435	0.179	0.310	0.750
	<i>Skdh-b</i>	0.090	0.091	0.097	0.071	0.024	0.000
	<i>Skdh-c</i>	0.490	0.477	0.468	0.750	0.667	0.250
Idh	<i>Idh-a</i>	0.920	0.996	0.843	0.964	1.000	1.000
	<i>Idh-b</i>	0.080	0.004	0.157	0.036	0.000	0.000
Pgi	<i>Pgi-a</i>	0.608	0.690	0.525	0.768	0.619	0.375
	<i>Pgi-b</i>	0.031	0.035	0.030	0.000	0.048	0.000
	<i>Pgi-c</i>	0.361	0.275	0.445	0.232	0.333	0.625
Enzyme		F_{is}^y					
Mdh2		-0.222****	-0.204***	-0.217****	-0.286	-0.290	-1.000
Mdh3		-0.209****	-0.051	-0.275****	-0.125	-0.290	NA ^s
Skdh		-0.152****	-0.186****	-0.150****	0.303	-0.013	-0.200
Idh		-0.174****	1.000****	-0.184****	-0.019	NA	NA
Pgi		0.016***	-0.032	0.082****	-0.286	-0.111	-0.500
Average		-0.132****					
Enzyme		H_o^x	H_s^w	H_t^v	F_{is}	F_{it}^u	F_{st}^t
Mdh2		0.573	0.405	0.423	-0.222	-0.165	0.046
Mdh3		0.267	0.223	0.234	-0.209	-0.152	0.047
Skdh		0.525	0.495	0.550	-0.152	-0.126	0.022
Idh		0.079	0.070	0.076	-0.174	-0.030	0.122
Pgi		0.546	0.474	0.502	0.016	0.063	0.048
Average		0.398	0.333	0.357	-0.132	-0.080	0.046

^zN = number of accessions.

^y F_{is} = deviations from Hardy-Weinberg equilibrium among individuals within populations.

^x H_o = observed heterozygosities.

^w H_s = expected heterozygosities within populations.

^v H_t = total diversity.

^u F_{it} = total deviations from Hardy-Weinberg among individuals.

^t F_{st} = deviation from Hardy-Weinberg among populations.

^sNA = missing data.

NUT SIZE. In Spain the average NNK was 110 nuts/kg (Table 3), but with variation ranging from 38–343 nuts/kg. There are also regional differences in NNK; with 119 nuts/kg in Asturias (smallest nuts), 113 nuts/kg in Castilla-León (El Bierzo), 108 nuts/kg in Galicia, 87 in Extremadura (Guadalupe) and, finally, 73 in Andalucía. Nuts from Andalucía weighed on average 14–17 g, the highest in Spain. Classifying all the accessions from Spain showed that 58% produced more than 100 nuts/kg; these accessions have a low market value. Only 7% of the accessions produced nuts averaging more than 15 g, and these were primarily from Andalucía. Chestnuts from Asturias were similar to those from Galicia in WLN, with only 2% to 6% of the accessions producing nuts over 15 g.

HARVESTING TIME. Early nut ripening is a useful genetic character for breeding. In Spain, harvest occurs from around 15 Sept. until mid-November. Only 16% of the accessions from Andalucía in southern Spain produced VEA nuts. In Asturias and Castilla-León, only between 2% to 5% of accessions produced EAR nuts, while in Galicia, late production is more common (43%).

NUT SHAPE. The most frequent shapes in Spain were the ellipti-

cal-short forms (110 < WRL < 120) found in 46% of accessions, the elliptical-triangular form (100 < WRL < 110) in 30% of accessions, and the elliptical-broad (WRL > 120) in 18% of accessions (classification according to Pereira-Lorenzo and Ramos-Cabrer, 2004). Triangular-shaped nuts (WRL < 100) were the least frequent category, and found in only 6% of accessions. The elliptical-short shape is predominant in northern Spain (Asturias, Castilla-León, and Asturias) while in the south, elliptical-triangular forms are more frequent.

CATKIN TYPE. In Spain, CLO is predominant (43% of the total accessions, 66% in Asturias, and 84% in Andalucía). In the high rainfall Galicia, CME is most frequent. Castilla-León appears to be a transition region between the CME of Galicia and the CLO of Asturias. Only CLO produces abundant pollen.

SPINE LENGTH. The length of the spines on burs is another character to distinguishing cultivars. BTS make harvesting (normally by hand) easier. In Galicia and Asturias, 19% and 11% of cultivars respectively had burs with BTS. In Andalucía, BTL were the most frequent (84%).

PDN. Non-divided nuts in *C. sativa* are thought to have resulted from selection (Breviglieri, 1955). In Spain, only 12% of

Table 3. Distribution of studied accessions of chestnut from Spain into five morphological and one phenological trait classes and mean values for quantitative traits classified by regions.

		Region of Spain											
		Asturias		Galicia		Castilla-León		Extremadura		Andalucía		Spain	
		accessions		accessions		accessions		accessions		accessions		accessions	
		(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)
Nuts (no./kg)													
<80		5	2	49	17	2	10	1	25	22	71	79	14
80–90		18	8	45	16	2	10	1	25	5	16	71	12
90–100		37	16	48	17	3	15	2	50	1	3	91	16
>100		177	75	141	50	13	65	0	0	3	10	334	58
Mean		119		108		113		87		73			
Weight of lateral nuts													
<10 g		93	39	111	39	9	45	0	0	3	10	216	38
10–15 g		139	59	158	55	10	50	4	100	9	30	320	56
>15 g		5	2	16	6	1	5	0	0	18	60	40	7
Mean		11		11		11		13		17			
Harvesting period													
Very early (VEA)		0	0	0	0	0	0	0	0	9	16	9	1
Early (EAR)		5	2	0	0	2	5	0	0	10	17	17	2
Medium (MED)		141	44	17	3	20	54	0	0	19	33	197	20
Late (LAT)		174	54	292	54	15	41	4	100	20	34	505	52
Very late (VEL)		3	1	234	43	0	0	0	0	0	0	237	25
Shape of the nuts													
Triangular		9	4	10	4	5	25	4	100	6	19	34	6
Elliptical-triangular		73	31	81	29	5	25	0	0	16	52	175	30
Elliptical-short		104	44	145	51	10	50	0	0	4	13	263	46
Elliptical-broad		51	22	48	17	0	0	0	0	5	16	104	18
Mean		114		113		107		94		106			
Male catkins													
Astaminate (CAS)		27	9	22	7	2	10	3	75	0	0	54	8
Brachystaminate (CBR)		18	6	64	19	2	10	0	0	1	3	85	13
Mesostaminate (CME)		52	18	179	54	8	40	0	0	4	13	243	36
Longistaminate (CLO)		190	66	69	21	8	40	1	25	26	84	294	43
Size of bur spines													
Short (BTS)		28	11	58	19	0	0	0	0	0	0	86	14
Medium (BTM)		161	61	206	67	20	100	0	0	5	16	392	63
Long (BTL)		76	29	43	14	0	0	4	100	26	84	149	24
Divided nuts (%)													
<12%		192	81	270	95	15	75	4	100	24	77	505	88
>12%		45	19	13	5	5	25	0	0	7	23	70	12
Mean		8		2		10		5		9			

the accessions produced more than 12% of PDN. Higher proportion were found in Andalucía and Castilla-León (23% and 25%), while in Galicia and Extremadura less than 5% of the accessions produced more than 12% of PDN.

CLASSIFICATION BY ISOZYMES, MORPHOLOGY AND PHENOLOGY. Using PCA we reduced 27 variables (15 corresponding to the six morphological traits and 12 to isotypes) to 11 PCs (PC1 to PC11) with an eigen value over 1. Those 11 PCs accounted for 78% of the total variance. Characteristics with highest eigen vectors were as follows: PC1, Mdh2100, Mdh2110, Idh100, Idh105, CME and CLO and LAT; PC2, Mdh395, Mdh3100, Pgi90 and Pgi100; PC3, Skdh100; PC4, BTM and BTL, VEA and EAR and NNK; PC5, BTM and BTL; PC6, Skdh97, CAS and NNK; PC7, BTS and BTM; PC8, Skdh97 and Skdh100 and EAR; PC9, Skdh97, WLR and BTS; PC10, CBR; and PC11, Pgi92 and CAS. The UPGMA cluster analysis carried out on 501 accessions produced 77 clusters when the dendrogram is cut at a Mahalanobis distance

of 0.6 (Fig. 2), 49 of them are shown in Table 4. Morphology confirmed most of the clusters with isozymes. Only two clusters were differentiated on morphological characteristics (WLR for cluster 12 and male catkins for cluster 24).

Possible synonymies were identified in Fig. 2 and Table 4 (synonyms are in parentheses): 1) cluster 1—‘Tempera’ and ‘Cabezuda’; 2) cluster 7—‘Amarelante’, ‘Abarcá’, ‘Anchas’, and ‘Desgrañadiza’; 3) cluster 7—‘Inxerta’ and ‘Porteliña’; 4) cluster 10—‘Luguesa’ and ‘Tarabelao’; 5) cluster 10—‘Presa’ and ‘Veiguiña’; 6) cluster 12—‘Injerta’ and ‘Gallego’; 7) cluster 12—‘Pareda’, ‘Padana’, ‘Paradesa’, and ‘Xidra’; 8) cluster 13—‘Chamberga’ and ‘Valduna’; 9) cluster 14—‘Negral’, ‘Courelá’, ‘Riá’, ‘Vileta’, and ‘Xabrega’; 10) cluster 15—‘Negral’ and ‘Vaquera’; 11) cluster 20—‘Galega’, ‘Colunga’, and ‘Zapatona’; 12) cluster 20—‘Largana’, ‘Nargana’, and ‘Parruca’; 13) cluster 22—‘Pelona’ and ‘Feltrona’; and 14) cluster 50—‘Negral’ and ‘Blanca’.

Fig. 2. Cluster of 501 accessions of chestnut (shown are the most relevant ones) with five loci and four morphological and one phenological traits. In parentheses the number of accessions clustered of each cultivar.

Table 4. Classification of Spanish chestnut cultivars and synonyms from five regions of Spain using five morphological and one phenological traits and genotypes described with four isoenzymes.

Cultivars (synonyms in parentheses)	Region	Accessions (no.)	Genotype	Cluster	Mdh2	Mdh3	Skdh	Idh	Pgi	Nuts (no./kg)	Harvesting period ^z	Nut shape ^y	Type of catkin ^x	Spine length ^w	PDN ^v (%)
Temperá (Cabezuda)	Galicia	5	1	1	ab	bb	bc	aa	aa	83	LAT	Ell-Short	CME	BTM	2
Corriente	Andalucía	1	1	72	ab	bb	bc	aa	aa	70	VEA	Ell-Short	CLO	BTM	13
Pelado	Galicia	3	2	2	aa	ab	bc	aa	aa	88	LAT,VEL	Ell-Broad	CME	BTM	2
Marela	Galicia	3	2	44	aa	ab	bc	aa	aa	131	LAT,VEL	Ell-Short	CAS	BTM	7
Amarelante (Abarcá, Anchas, Galicia Desgrañadiza)	Galicia	12	3	7	ab	ab	ac	aa	cc	88	LAT,VEL	Ell-Short	CME	BTM	1
Inxerta (Porteliña)	Galicia	5	4	7	aa	ab	bc	aa	cc	76	LAT,VEL	Ell-Tr	CME	BTM	0
Injerta	Extremadura	1	5	8	ab	bb	ac	aa	cc	78	LAT	Triang	CLO	BTL	0
Luguesa (Tarabelao)	Galicia	4	6	10	aa	bb	ab	aa	cc	97	LAT,VEL	Ell-Short	CME	BTM	1
Presa (Veiguiña)	Galicia	4	7	10	aa	bb	cc	aa	ac	97	LAT,VEL	Ell-Short	CME	BTM	1
Galega	Asturias	2	7	16	aa	bb	cc	aa	ac	130	MED	Ell-Tr	CLO	BTM	5
Injerta	Asturias	5	8	12	aa	bb	ac	aa	aa	152	MED,LAT	Ell-Tr	CLO	BTM	13
Injerta (Gallego)	Castilla-León	5	8	12	aa	bb	ac	aa	aa	141	MED,LAT	Triang	CME	BTM	7
Pareda (Padana, Paradesa, Xidra)	Asturias, Castilla-León, Galicia	34	8	12	aa	bb	ac	aa	aa	135	MED,LAT, VEL	Ell-Tr	CME	BTM	6
Rapuca	Asturias	3	8	61	aa	bb	ac	aa	aa	125	MED	Ell-Short	CBR	BTS	3
Chamberga (Valduna)	Asturias	16	9	13	ab	bb	ac	aa	aa	96	MED,LAT	Ell-Broad	CLO	BTM	2
Valduna2	Asturias	3	9	23	ab	bb	ac	aa	aa	99	MED,LAT	Ell-Short	CLO	BTL	4
Crespa	Asturias	3	10	13	bb	bb	aa	aa	ac	164	MED,LAT	Ell-Short	CLO	BTM	4
Negrera	Asturias	2	11	13	aa	bb	aa	aa	aa	115	MED,LAT	Ell-Short	CLO	BTM	4
Roxa	Asturias	2	12	13	ab	bb	aa	aa	cc	135	MED,LAT	Ell-Short	CLO	BTM	12
Vizcaína	Asturias	2	13	13	ab	bb	aa	aa	aa	111	MED,LAT	Ell-Tr	CLO	BTM	7
Grúa	Asturias	4	13	20	ab	bb	aa	aa	aa	90	MED,VEL	Ell-Broad	CLO	BTL	7
Garrida	Galicia	3	13	56	ab	bb	aa	aa	aa	74	LAT,VEL	Ell-Short	CBR	BTM	2
Negral (Courelá, Riá, Vileta, Xabrega)	Galicia, Castilla-León	15	14	14	ab	ab	aa	aa	ac	103	EAR,MED, LAT	Ell-Short	CLO	BTM	2
Pelona	Asturias	2	15	14	ab	ab	ac	aa	aa	173	MED,LAT	Ell-Tr	CLO	BTM	3
Pelona (Feltrona)	Asturias	4	15	22	ab	ab	ac	aa	aa	117	MED,LAT	Ell-Short	CLO	BTL	1
Negral (Vaquera)	Castilla-León, Asturias	5	16	15	ab	ab	cc	aa	ac	98	MED,LAT	Ell-Short	CLO	BTM	7
Arial	Galicia	2	17	16	aa	bb	cc	aa	aa	81	LAT,VEL	Ell-Tr	CLO	BTM	2
Navexa	Asturias	3	17	16	aa	bb	cc	aa	aa	104	LAT	Ell-Short	CLO	BTM	6
Vegamesada	Asturias	2	17	16	aa	bb	cc	aa	aa	92	MED,LAT	Ell-Short	CME	BTM	6
Planta Alajar	Andalucía	4	17	24	aa	bb	cc	aa	aa	71	MED,LAT	Triang	CME	BTL	11

Table 4. Continued on next page.

Table 4. Continued from previous page.

Cultivars (synonyms in parentheses)	Region	Accessions (no.)	Genotype	Cluster	Mdh2	Mdh3	Skdh	Idh	Pgi	Nuts (no./kg)	Harvesting period ^z	Nut shape ^y	Type of catkin ^x	Spine length ^w	PDN ^v (%)
Pilonga	Andalucía	2	17	73	aa	bb	cc	aa	aa	68	EAR,ME	Ell-Tr	CLO	BTL	9
											D,LAT				
Corriente	Andalucía	1	17	74	aa	bb	cc	aa	aa	58	VEA,EAR	Ell-Broad	CLO	BTM	20
Pilonga	Andalucía	4	17	75	aa	bb	cc	aa	aa	63	VEA,EAR	Ell-Tr	CLO	BTL	8
Bermella	Galicia	2	18	17	ab	bb	ac	ab	ac	145	LAT,VEL	Ell-Short	CME	BTM	11
Picona	Galicia	2	19	17	aa	bb	ac	ab	aa	119	LAT,VEL	Ell-Short	CME,	BTM	14
													CLO		
Serodia	Galicia	2	19	17	aa	bb	ac	ab	aa	101	LAT,VEL	Ell-Broad	CLO	BTM	1
Rapada	Galicia	8	19	32	aa	bb	ac	ab	aa	103	LAT,VEL	Ell-Short	CME	BTS	4
Praga Dafora	Galicia	2	19	66	aa	bb	ac	ab	aa	98	LAT,VEL	Ell-Short	CBR	BTM	1
Ventura	Galicia	5	20	19	aa	ab	bc	ab	aa	90	LAT	Ell-Tr	CME	BTM	0
Argua	Asturias	2	21	20	aa	bb	ab	aa	ac	95	LAT,VEL	Ell-Broad	CLO	BTL	5
Galega (Colunga, Zapatona)	Asturias	7	22	20	ab	bb	ab	aa	aa	93	MED,LAT	Ell-Short	CLO	BTL	8
Boroña	Asturias	1	22	72	ab	bb	ab	aa	aa	111	LAT	Ell-Short	CLO	BTM	4
Largaña (Nargana, Parruca)	Asturias	8	23	20	ab	bb	aa	aa	ac	123	MED,LAT	Ell-Short	CLO	BTL	5
Verata	Extremadura	3	23	54	ab	bb	aa	aa	ac	90	LAT	Triang	CAS	BTL	6
Llanisca	Asturias	4	24	21	bb	bb	ab	aa	cc	123	LAT	Ell-Broad	CLO	BTL	10
Comisaria	Andalucía	1	25	22	aa	ab	cc	aa	ac	69	LAT	Ell-Tr	CLO	BTL	4
Pelona	Andalucía	1	26	22	aa	ab	ac	aa	ac	72	MED,LAT	Ell-Tr	CLO	BTL	8
Vázquez	Andalucía	1	27	22	ab	ab	bc	aa	ac	68	MED,LAT	Ell-Short	CLO	BTL	0
Rapuca	Asturias	2	28	29	aa	ab	ac	aa	ac	134	MED,LAT	Ell-Short	CME	BTS	6
Verde	Galicia	3	28	45	aa	ab	ac	aa	ac	105	VEL	Ell-Broad	CAS	BTM	3
Verde	Galicia	1	28	47	aa	ab	ac	aa	ac	97	VEL	Ell-Broad	CAS	BTS	1
Doriga	Asturias	2	29	23	ab	bb	cc	aa	ac	154	MED	Ell-Short	CLO	BTL	8
Doriga	Asturias	2	29	51	ab	bb	cc	aa	ac	134	MED,LAT	Ell-Short	CAS	BTM	13
Verde	Asturias	2	29	51	ab	bb	cc	aa	ac	147	MED,LAT	Ell-Short	CAS	BTM	15
Doriga	Asturias	2	29	52	ab	bb	cc	aa	ac	141	MED,LAT	Ell-Short	CAS	BTL	9
Helechal	Andalucía	1	30	23	ab	bb	ac	aa	ac	94	MED,LAT	Ell-Tr	CLO	BTL	8
Tomasa	Andalucía	1	31	24	ab	bb	cc	aa	aa	46	MED,LAT	Triang	CLO	BTL	14
Temprana	Andalucía	3	31	75	ab	bb	cc	aa	aa	61	VEA,EAR	Ell-Broad	CLO	BTL	12
Dieguina	Andalucía	2	32	25	ab	ab	bc	ab	ac	87	MED,LAT	Ell-Tr	CME,	BTL	8
													CLO		
Pelona	Andalucía	1	33	31	aa	ab	aa	aa	ac	140	MED,LAT	Triang	CLO	BTL	3
Loura	Galicia	1	34	37	aa	ab	bc	aa	bc	78	LAT,VEL	Ell-Broad	CBR	BTM	0
Loura	Galicia	4	34	38	aa	ab	bc	aa	bc	82	LAT,VEL	Ell-Broad	CME	BTM	1

Table 4. Continued on next page.

Table 4. Continued from previous page.

Cultivars (synonyms in parentheses)	Region	Accessions (no.)	Genotype	Cluster	Mdh2	Mdh3	Skdh	Idh	Pgi	Nuts (no./kg)	Harvesting period ^z	Nut shape ^y	Type of catkin ^x	Spine length ^w	PDN ^v (%)
Verde	Galicia	1	35	46	aa	ab	ac	aa	bc	136	LAT,VEL	Ell-Broad	CAS	BTM	2
Blanca	Galicia	1	36	48	aa	bb	ac	aa	cc	212	VEL	Ell-Tr	CAS	BTS	34
Verde	Asturias	1	37	49	aa	bb	ac	aa	bc	137	LAT	Ell-Broad	CAS	BTS	7
Blanca	Galicia	2	38	50	ab	bb	aa	ab	ac	125	LAT,VEL	Ell-Broad	CAS	BTM	1
Negral (Blanca)	Galicia	2	38	50	ab	bb	aa	ab	ac	103	LAT,VEL	Ell-Broad	CAS	BTM	0
Puga do Receiro	Galicia	1	38	66	ab	bb	aa	ab	ac	127	LAT,VEL	Ell-Short	CBR	BTM	1
Rapega	Castilla-León	1	39	51	aa	bb	ac	aa	ac	100	MED,LAT	Ell-Short	CAS	BTM	13
Verdello	Castilla-León	1	39	51	aa	bb	ac	aa	ac	123	MED,LAT	Ell-Short	CAS	BTM	25
Longal	Galicia	5	39	62	aa	bb	ac	aa	ac	118	LAT,VEL	Triang	CBR	BTL	0
Raigona	Galicia	7	40	59	ab	ab	ac	aa	ac	128	LAT	Ell-Short	CBR	BTM	10
Puga do Bolo	Galicia	2	40	61	ab	ab	ac	aa	ac	89	VEL	Ell-Tr	CBR	BTS	0
Famosa	Galicia	5	41	64	aa	bb	ac	ab	cc	86	LAT,VEL	Ell-Short	CBR	BTL	0
Famosa	Galicia	1	42	65	ab	bb	ac	aa	bc	99	LAT,VEL	Ell-Short	CBR	BTL	0
Puga do Bolo	Galicia	1	43	66	aa	bb	aa	ab	ac	104	VEL	Ell-Short	CBR	BTM	0
Salnesa	Galicia	1	44	66	aa	bb	ac	ab	ac	168	LAT	Ell-Short	CBR	BTM	0
Xabrega	Galicia	2	45	66	aa	bb	aa	ab	aa	120	VEL	Ell-Short	CBR	BTM	0
Marelo	Castilla-León	2	46	67	ab	ab	cc	aa	bc	133	EAR,ME	Ell-Short	CBR	BTM	15
											D,LAT				
Verde	Asturias	2	47	70	bb	bb	ac	aa	aa	82	MED	Ell-Broad	CLO	BTM	55
Miguelina	Asturias	1	48	76	aa	bb	aa	aa	ab	112	EAR,ME	Ell-Short	CAS	BTM	19
											D,LAT				
Miguelina	Asturias	1	49	77	ab	bb	ac	aa	ab	102	MED	Ell-Short	CAS	BTM	34

^zVEA = very early, EAR = early, MED = medium, LAT = late, VEL = very late.

^yTriang = triangular, Ell-Tr = elliptical-triangular, Ell-Short = elliptical-short, Ell-Broad = elliptical-broad.

^xCAS = astaminate, CBR = brachystaminate, CME = mesostaminate, CLO = longistaminate.

^wBTS = short, BTM = medium, BTL = long.

^vPDN = percentage of divided nuts.

A total of 152 Spanish cultivars were classified into 58 cultivars of major importance and 94 with minor importance, 18 of which had high intracultivar variation. A total of 14 groups of possible synonymies were detected involving 37 cultivars. We observed six groups in Galicia containing 14 cultivars, one group in Castilla-León (El Bierzo) with two cultivars, four groups in Asturias with 10 cultivars, one group in Asturias and Castilla-León (El Bierzo) with two cultivars, and two groups in Asturias, Castilla-León (El Bierzo) and Galicia with nine cultivars.

Although 18 out of 152 cultivars classified had high intracultivar variation (12%), the cultivars most intensively studied (three or more accessions from different localities), did not vary greatly. For instance, in Galicia and Asturias, 58% and 57% of accessions of a specific cultivar, respectively, were grouped in the same cluster. This suggests that a particular clone has been propagated for many years in each of these regions. 'Parede' clus-

tered with 23 accessions out of 29 studied in Asturias (79%) and coincidentally with six out of nine studied in Galicia (67%).

Discussion

This study classified 152 Spanish chestnut cultivars into 58 of major importance and 94 of minor importance. A total of 14 groups of possible synonymies were detected involving 37 cultivars. All isotypes in this study had previously been found in Galicia (Pereira-Lorenzo et al., 1996b), suggesting that the whole population of Spanish cultivars came from a common pool. The cultivars of Galicia and Asturias can be defined as a continuum of common isotypes and cultivars and representing an ancestral gene pool. The chestnuts in Galicia (300 accessions studied by isozymes) are a good representation of the entire Spanish chestnut population (617 accessions in total) and represent a region with the highest variation in Spain.

The excess of heterozygotes found in Galician chestnuts was similar to the studies by Stilwell et al. (2003), who used five isozymes on five natural populations of *C. dentata* in Virginia. These authors explained excess heterozygosity by the selection of heterozygous breeding material and by an absence of new recruits from other populations. These *C. dentata* populations were over 70 years old; the orchards studied here are at least 300 years old. Nevertheless, the excess of heterozygotes observed might have resulted from patterns similar to those described by Stilwell et al. (2003). Subsequent asexual propagation through grafting would maintain this situation. The F statistics indicated a small differentiation among populations ($F_{st} = 0.046$); this could be explained by the movement of clonal cultivars, such as 'Paredé', between more than one region. Subpopulations isolated in southern Spain had lower variation.

The importance of chestnut cultivation reduces from the north to the south of Spain, as indicated by the lower number of cultivars in southern Spain. It may also be significant that there are major physical barriers between the different areas. In Andalucía the cultivars are less variable and fall into fewer groups than those in provinces of northern Spain; this could be due to the region's geographic isolation. However, cultivars from Castilla-León are grouped with the principal cultivars of Asturias and Galicia, which indicates that selection and spread in these regions were less affected by geographical location. This is reflected in little differentiation among populations ($F_{st} = 0.046$).

Morphological characters were extremely useful for identifying cultivars and, when used in combination with isozymes, have considerably increased cultivar discrimination. The most important morphological characteristic of chestnuts for the market is the size of the nuts. It is also a useful character, as reported earlier, for the selection, management, and classification of chestnut cultivars (Oraguzie et al., 1988). Of all Spanish accessions, 58% produced more than 100 nuts/kg. Removing this low-value characteristic should be of high priority in breeding projects to produce new cultivars. Harvesting time is the second most important characteristic for marketing chestnuts. Yet only between 2% and 16% of the accessions from Andalucía, Asturias, and Castilla-León produced EAR nuts and in Galicia, LAT nuts are common (43%). However, *C. sativa* tends to be harvested later than Asiatic species. Nut shape is important for distinguishing cultivars. Some cultivar names, such as Longal, are given based on shape (Pereira-Lorenzo, 1996a, and present data). Male catkin types and length of spines on burs are also useful characters to distinguish cultivars. We included in this study information on PDN, which is a disadvantage for nut peeling. In Spain up to 25% of accessions, depending on region, had the detrimental character of producing more than 12% of PDN.

The Iberian Peninsula, in particular northern and northwestern Spain, is an important center of genetic diversity of chestnut, and a region where cultivars have been selected for centuries. Material from this region is a valuable genetic resource for future chestnut breeding and selection.

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