Theor Appl Genet (1994) 87:829–836

THEORETICAL AND APPLIED GENETICS

On the origin of Spanish two-rowed barleys

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Received: 20 May 1993 / Accepted: 16 June 1993

Abstract. To investigate the phylogenetic origin of Spanish two-rowed barleys, we studied 44 accessions of old land-races both morphologically and biochemically to ascertain their similarity with 51 entries of old cultivars and land-races of widespread origin across Europe. They were also compared with 20 accessions of *Hordeum spontaneum* from the Mediterranean basin and other regions of its distribution range, 14 accessions of Moroccan cultivated six-rowed barley land-races, and different six-rowed Spanish and two-and six-rowed European cultivars. CM- (trypsin inhibitors and subunits of the barley tetrameric α-amylase inhibitor) proteins and hordeins, all of which are endosperm proteins, were used as biochemical markers. The appearance of separate clusters of the Spanish barleys in the numerical classifications for both protein systems as a result of the existence of characteristic gene combinations that do not exist in entries from other origins permitted us to postulate the existence of local ancestors for most of the Spanish two-rowed barleys studied, and, therefore, a possible *in situ* domestication.

Key words: Centre of origin – Wild barley – Hordeins – CM-proteins

Introduction

The geographical origin of cultivated barley (*Hordeum vulgare* L.) is a subject of debate. The most accepted theory (see Harlan 1992) states that barley is a monocentric crop that originated in the Fertile Crescent, i.e., it has a unique center of origin, where it was derived from its wild ancestor *Hordeum spontaneum* C. Koch when Neolithic man selected those spikes with tough rachis. However, the discovery of wild barley in Morocco (Molina-Cano and Conde 1980; Molina-Cano et al. 1982) and the Qinghai-Xizang plateau in China (Xu 1982) enlarged considerably its distribution limits, and these findings led Molina-Cano et al. (1987) to postulate Morocco as a possible center of origin of cultivated barley. Thus, cultivated barley could be a multicentric crop. This latter study also confirmed Wiebe's (1968) suggestion that Spanish six-rowed barleys were of Moroccan origin.

The collection, and agronomic characterization of Spanish barley land-races has been carried out for many years (e.g., Alvarez-Peña 1952; Villena 1955; Molina-Cano 1977; Jouve et al. 1990), but no information has yet been gathered on the true origin of Spanish two-rowed barleys. We therefore decided to investigate the origin of these barleys by studying two different groups of grain proteins and a set of morphological characters.

CM-proteins are a group of endosperm proteins that are soluble in chloroform-methanol mixtures (Salcedo et al. 1982) and which belong to the α-amylase-trypsin inhibitor family (Garcia-Olmedo et al. 1987, 1992). Some of them (CMc, CMe) are active against trypsin (Barber et al. 1986), while others (CMa, CMb, CMd) are the subunits of the tetrameric inhibitors of insect α-amylases (Sánchez-Monge et al. 1986; Moralejo et al. 1993a, b). The CM-proteins show a very low intra-specific variability in both *H. vulgare* and *H. spontaneum* (Salcedo et al. 1984; Molina-Cano et al. 1987). In contrast B-, C- and D-hordeins, the main storage proteins of the barley seed, show a high intergenotypic variation and have been used as molecular

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Communicated by J. Mac Key
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markers for the identification of barley cultivars (Doll and Brown 1979; Shewry et al. 1978a,b, 1979, 1983).

While genetic variation for morphological characters is very high in barley (e.g., Nilan 1964), we considered their study to be necessary for broadening the picture of the existing differences among the barley material studied.

Materials and methods

Plant material

Forty-four accessions of Spanish two-rowed land races (Table 1) were obtained from the Banco Nacional de Germoplasma (INIA, Madrid, Spain), Aula Dei Experimental Station (Zaragoza, Spain), and the barley germplasm collection of the Spanish brewery La Cruz del Campo as bulk grain. After one season of observation in 1989–1990, spikes were randomly taken, and bulk grain was harvested from each population. Fifty-one accessions of cultivars and land populations of other origins, mostly European (Table 1), were obtained from two different sources: the above-mentioned collection of La Cruz del Campo and the USDA Small Grains Collection kindly supplied by Dr. H. Bockelman. In addition, a set of 18 wild barley accessions studied by Molina-Cano et al. (1987) that covered almost the whole Fertile Crescent and Mediterranean basin and 2 more accessions of *H. spontaneum* from Crete and Greece were included. These were kindly supplied by Prof. C. Gómez-Campo (Madrid) and Dr. C. Theoulakis (Thessaloniki).

Table 1. Geographical origin of the barley accesions analyzed

Country of origin	Number of accessions	Source of the seed[a]
Austria	2	USDA
Bulgaria	1	USDA
Czechoslovakia	12	USDA
Denmark	1	USDA
France	3	USDA
Germany	6	USDA
Great Britain	2	USDA
Hungary	3	USDA
Morocco	1	BNG
Romania	3	USDA
Spain	36	BNG
Spain	8	LCC
Turkey	13	USDA
Unknown	4	BNG

[a] USDA, United States Department of Agriculture World Collection; BNG, Banco Nacional de Germoplasma (INIA, Madrid, Spain). Aula Dei Experimental Station (Zaragoza, Spain); LCC, Collection of the brewery, La Cruz del Campo, Spain

Field methods and observation of morphological characters

The material was sown in the field on a spike to row basis with two spikes per accession. The plants obtained were first for observation of morphological and phenological characters and, when harvested, for morphological characters of the spike and grain. Seed was also kept for the biochemical studies. The characters studied are listed in Table 2. The classification system used was that of Molina-Cano (1976) and Molina-Cano and Elena-Rosselló (1978).

Studies on CM-proteins

The extraction and two-dimensional electrophoretic fractionation of barley CM-proteins was carried out essentially as described by Salcedo et al. (1984). Single kernels from the progenies of individual spikes of each studied accession were delipidated with petroleum ether (B.P. 50°–70 °C; 1 × 10 vol for 1 h) and then extracted with chloroform-methanol (2:1 v/v; 2 × 10 vol for 1 h). The extracts were fractionated by two-dimensional gel electrophoresis: the first dimension consisted of isoelectrofocusing (IEF) on preformed pH gradients (ampholines 4–9), and the second one, a starch-gel electrophoresis (SGE; pH 3.2). Gels were stained with 0.05% nigrosine in methanol-water-acetic acid (5:5:1 by vol). To ascertain the relative positions of different protein variants in the two-dimensional maps, the electrophoresis of mixtures of the appropriate samples was carried out.

Studies on hordeins

Sodium dodecyl sulphate poliacrylamide gel electrophoresis (SDS-PAGE) was performed using a modification of the method described by Laemmli (1970). Hordeins were extracted with 10% (v/v) mercaptoethanol, 4% (v/v) SDS, 10% glycerol, 125 m*M* Tris-HCl, pH 6.8, and 0.08% bromophenol blue. The stacking gel layers contained 4% (w/v) acrylamide and 0.11% (w/v) *NN'*-methylene-bisacrylamide (BIS); the separating gels contained 12% (w/v) acrylamide and 0.024% (w/v) BIS. The gels were run for 6 h on a (15 × 20 × 1 cm) gel at 30 mA constant current and then fixed and stained in 40% methanol, 10% trichloroacetic acid (TCA), and 0.1% Coomassie BB R250.

Table 2. List of characters used

Vegetative phase	Spike	Kernel
Hairiness on the leaf sheaths	Relationship between awn length and spike length	Hairiness of the rachuilla
Juvenile habit at tillering	Glume length related to kernel length	Lodicule size
Flag-leaf habit at heading	Hairiness of the glume	Hairiness of ventral furrow
Anthocyanin on the flag-leaf auricles	Collar shape	Spicules on lateral nerves of the lemma
Earliness in heading	Rachis shape	Color of the cariopsis
	Length of rachis internodes	Rachilla colour
		Relative position of sterile spikelets
	Marginal hairiness of rachis internodes	Length of sterile spikelets
		Hairiness of sterile spikelets

Multivariate statistical methods

Three original data matrices were obtained after the observation and coding of the studied characters: (1) morphological and phenological characters; (2) CM-protein variants; (3) hordein patterns. The morphological characters were coded according to Molina-Cano (1976) and Molina-Cano and Elena-Rosseló (1978). The CM-proteins were coded as follows: each variant as 1 when present and as 0 when absent. A different code, from 1 to 13 (B-hordeins) and from A to I (C-hordeins), was given to each of the hordein patterns present, with each pattern consisting of a determined number of bands with different mobilities. This pattern definition used is similar to that of Shewry et al. (1979).

2M Block Clustering, with Jaccard's distance as the similarity coefficient (Dixon et al. 1990) was used for constructing the dendrograms.

Results

Morphological and phenological characters

The distribution of the Spanish and European barleys for the different character-states studied (data not shown) was very similar, indicating a close morphological resemblance amongst them. In fact, in the dendrogram constructed (not shown) there was a complete mixture of the different accessions regardless of their geographic origin. Consequently, the distinct clusters formed did not bear any geographical meaning. This was in contrast with the results of Molina-Cano (1977), which showed that the Spanish two-rowed material studied clearly clustered apart from the European cultivars used as controls.

CM-proteins

The two-dimensional maps of the different cultivars showing the allelic variants found in this study are summarized in Fig. 1. The standard phenotype, that of the 'Zephyr' and 'Bomi' barleys, consists of five components: CMa-1, CMb-1, CMc-1, CMd-1 and CMe-1 (Salcedo et al. 1984). Figure 2 gives a comparison of Spanish and European accessions for the percentages of genetic variants found in CM-proteins. It can be observed that the components CMa, CMc, and CMd were invariant in all of the material studied.

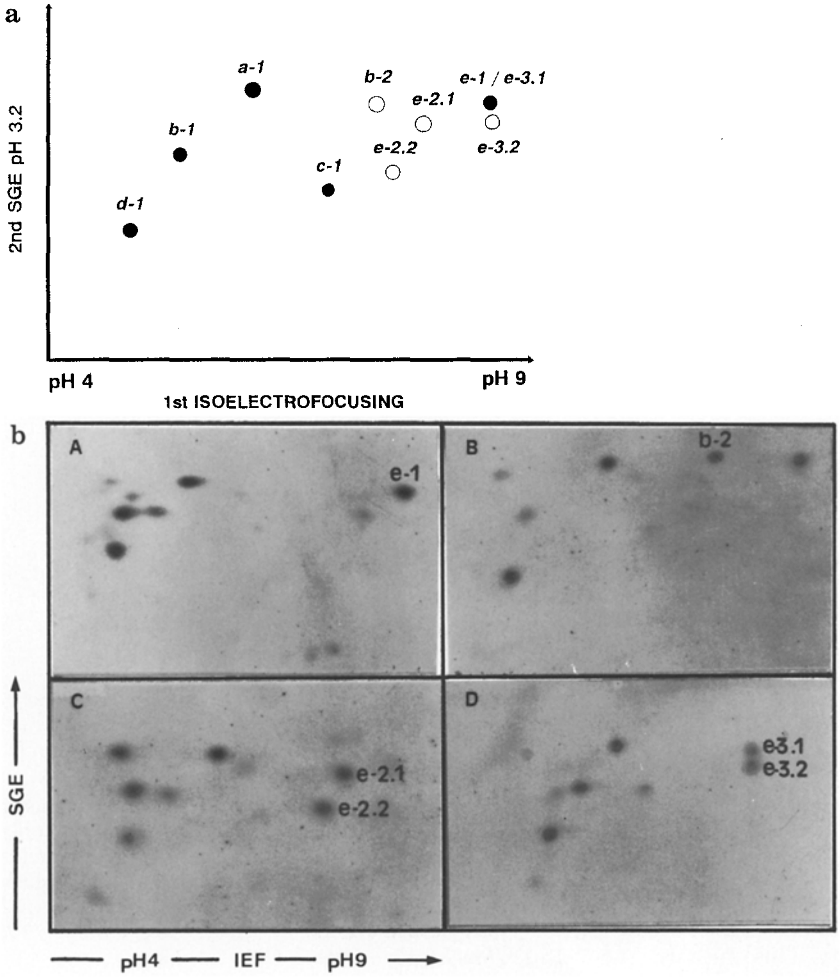

Fig. 1. **a** Composite diagram showing relative positions in the two-dimensional map and designation of all the observed CM-protein variants (*black spots* correspond to the standard phenotype of barley cvs 'Zephyr' and 'Bomi'; *open circles* to the variants). **b** *A* Two-dimensional map of the gel corresponding to cv 'Bomi', *B* allelic variant CMb-2, *C* allelic variants CMe-2.1-2.2, *D* allelic variants CMe-3.1-3.2

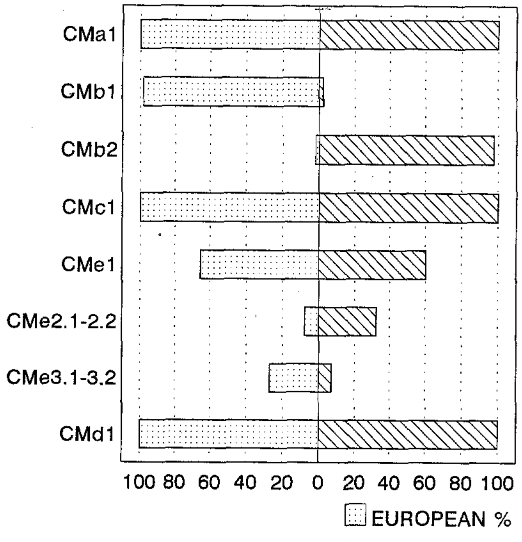

Fig. 2. Percentages of variants of CM-proteins found in the studied material

Of all Spanish barleys analyzed 77% were of genotype CMb-2. This variant has been observed in Moroccan accessions of *H. spontaneum*, in some Spanish six-rowed cultivars, and in the French cvs 'Hatif de Grignon' and its derivatives (all six-rowed) and 'Porthos' (Molina-Cano et al. 1987), and can, therefore, be considered to be a western Mediterranean variant. This allelic variant has been isolated, purified, and sequenced, and its inhibitory capacity against *Tenebrio molitor* α-amylase studied (Moralejo et al. 1993a). A single, but significant, difference in the first 23 amino acid residues was found between CMb-1 and 2, but there were no differences in their inhibitory capacity.

The standard protein CMe-1 appears in 63% of the material studied, and 4 variants, CMe-2.1, CMe-2.2, CMe-3.1, and CMe3.2, made up the rest of accessions. The variants CMe-2.1 and 2.2 are jointly inherited and are present in 19 accessions, 14 of them of Spanish origin. This variant (formerly called CM-2.2'; Salcedo et al. 1984) has been found in 'Hatif de Grignon' and its derivatives, Moroccan accessions of six-rowed barleys, and *H. spontaneum* (Molina-Cano et al. 1987). In the present work this variant also appeared in some plants of the *H. spontaneum* population from Crete. This is also, therefore, a variant of almost exclusive western Mediterranean origin. The variants CMe-3.1 and 3.2 always appeared together in the two-dimensional electrophoretic maps, being present in 18% of the material studied. Although CMe-3.1 overlaps with CMe-1 in the two-dimensional electrophoretic maps (see Fig. 1a), Moralejo et al. (1993b) have shown that both variants differ in their molecular size when subjected to SDS-PAGE and in their anti-trypsin activity. This study is the first to find CMe-3.1 and 3.2. As they are distributed almost randomly within the accessions, there is no discrimination among geographical origins. Only 3 Spanish accessions presented these variants, which were also present in genotypes of the *H. spontaneum* populations from Greece and Crete, the latter having been shown to be heterogeneous with respect to the CM-proteins.

The variants at locus CMe referred to above have been isolated and characterized, and their trypsin inhibitory capacity tested (Moralejo et al. 1993b). In all of them, except CMe-3.1, differences appeared in their *N*-terminal sequences if compared to CMe-1, and all were inhibitory against bovine trypsin but not against the α-amylases of storage cereal pests. Variant CMe-2.1 showed, however, residual activity against α-amylase from *T. molitor*.

The results of the cluster analysis on the data collected on the CM-proteins are presented in Fig. 3, where the five clusters formed are shown. Cluster 1 is composed of 42 accessions with the standard genotype CMa-1, CMb-1, CMc-1, CMd-1, and CMe-1. There are only 4 Spanish accessions in it. Cluster 2 consists of 22 entries with genotype CMa-1, CMb-2, CMc-1, CMd-1, and CMe-1. All entries are of Spanish origin, except for 1 from Turkey. Cluster 3 holds 15 accessions with genotype CMa-1, CMb-1, CMc-1, CMd-1, and CMe-3.1-3.2 and includes only 1 Spanish accession and 2 *H. spontaneum* populations from Greece and Crete. The genetic constitution of cluster 4 is CMa-1, CMb-2, CMc-1, CMd-1, and CMe-2.1-2.2. It is formed by 12 Spanish accessions. Cluster 5, with genotype CMa-1,

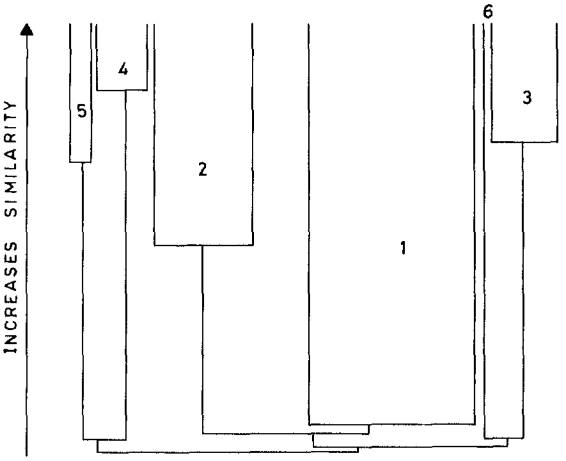

Fig. 3. Dendrogram of data on the CM-proteins. For an easier interpretation, all clusters have been simplified by drawing only the limiting branches. Taking into account that the proteins CMa, CMc and CMd were invariant within the material studied, the genotypes of the different clusters are: *1* CMb-1, CMe-1; *2* CMb-2, CMe-1; *3* CMb-1, CMe-3.1, 3.2; *4* CMb-2, CMe-2.1, 2.2; *5* CMb-1, CMe-2.1-2.2; *6* CMb-2, CMe-3.1, 3.2

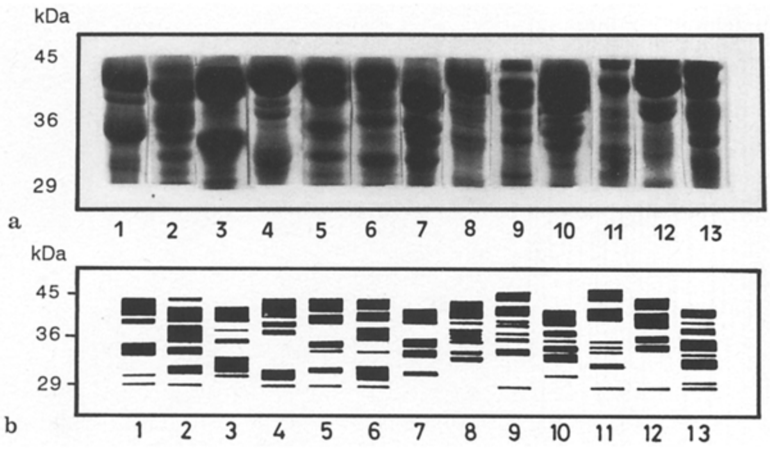

Fig. 4. **a** SDS-PAGE patterns of the B-hordeins found in the material studied. Only the appropriate part of the gel is shown. **b** Diagrammatic representation of the bands shown in **a**

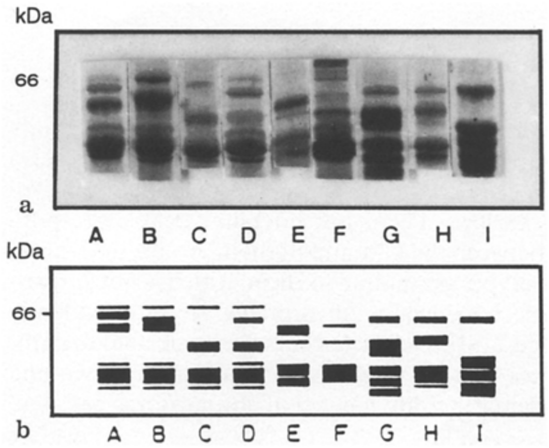

Fig. 5. **a** SDS-PAGE patterns of the C-hordeins found in the material studied. **b** Diagrammatic representation of the bands shown in **a**

CMb-1, CMc-1, CMd-1, and CMe-2.1-2.2, is formed by 5 entries: 2 from Spain, the 2 *H. spontaneum* from Greece and Crete, and 1 of unknown origin, although it was catalogued as being collected in Spain.

Only one entry clustered apart from the referred groups. This had the genotype CMa-1, CMb-2, CMc-1, CMd-1, and CMe-3.1-3.2, and it is also of Spanish origin.

Hordeins

The different banding patterns obtained with B- and C-hordeins are depicted in Figs. 4 and 5, respectively. The patterns of the D-hordeins, because of their simplicity, are not presented. There are 13 different polypeptide patterns of the B-hordeins, named 1–13 in Fig. 4, and 9 of the C-hordeins, called A–I in Fig. 5.

B-hordein pattern 1 is characteristic of Spain (20 of 28 accessions are of Spanish origin), whereas the remaining patterns are heterogeneous with regard to geographic origin. Within the C-hordeins, patterns A and B were fairly typical of Spanish barleys: 60% of the entries of this origin show these banding patterns. The D-hordein electrophoretic patterns were very simple, with only two alternative bands being present in each accession. The most common pattern, called D1, was shown by 83 out of the 95 entries, with the remaining ones showing the D2 pattern. Only 1 Spanish accession carried the D2 pattern.

Clustering of the studied entries on the basis of hordein patterns is presented in Fig. 6. Many grouping emerge, although only clusters 1 to 4 are relevant for our purposes. Cluster 1 is made up totally of Spanish entries, while 10 out of 13 genotypes in cluster 2 are Spanish. In cluster 3, 6 out of 9 accessions are from Spain, and in cluster 4 all 12 entries are of Spanish origin.

In summary, 73% of Spanish barleys grouped together in clusters 1–4, with the remaining ones being distributed among the other clusters. It can, therefore, be concluded that there are hordein patterns typical of barleys from Spain.

Discussion

Evidence of a Spanish group of germ plasm of two-rowed barley

Groups composed chiefly or totally of Spanish accessions were formed with regard to both CM-proteins and hordeins. Thus, the gene combination CMb-2, CMe-1 is exclusive to Spanish entries, with the exception of 1 entry from Turkey, whereas the genotype CMb-2, CMe-2.1-2.2 is shared by Spanish, French, and Moroccan entries. Therefore, both groups can be considered to be of western Mediterranean origin. On the

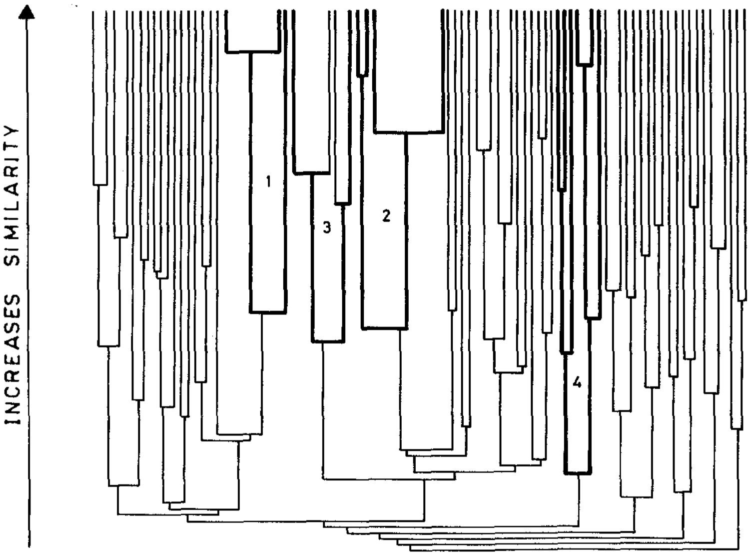

Fig. 6. Dendrogram of the hordein data. The relevant clusters have been simplified for easier interpretation

other hand, hordein clusters 1–4 (Fig. 6) are mainly made up of Spanish germ plasm, i.e., there are also hordein gene groupings characteristic of Spanish two-rowed barleys. There are no biunivocal correspondence between the CM and hordein groups in terms of the genotypes pertaining to them, which is not surprising since both groups of proteins are independently inherited. CMb and CMe genes are associated to chromosomes 4 and 3, respectively, whereas the B-, C- and D-hordein genes are located in chromosome 5.

Implications relevant to the origin of Spanish two-rowed barleys

The geographical significance of these groups allows us to formulate some hypotheses on the origin of the Spanish barleys studied.

The almost exclusive specificity of gene combination CMb-2, CMe-1 to Spain (the Turkish exception could be due to a mutation event uncommon within the area since all other Turkish entries were of genotype CMb-1) and the absence of any *H. spontaneum* with this genotype (see also Molina-Cano et al. 1987) suggests an ancient *in situ* domestication with a subsequent disappearance of the putative wild barley ancestor. The present evidence does not contradict this hypothesis, and the ecological similarity between Greece (with actual survival of *H. spontaneum*) and Spain suggests the existence of wild barley populations in the Iberian Peninsula in Neolithic times. The disappearance of *H. spontaneum* from the Iberian Peninsula could have happened in a manner parallel to the one postulated for it in Cyprus by Hadjichristodoulou (1992), that in the natural pasture lands of this island it was eliminated by thousands of years of overgrazing. Strabo (Greek geographer and historian who died in 23 A.D.) in his *Geographical Sketches* describes Iberia as being almost completely covered by forests and mountains (cited by Mangas 1980). It is, however, a well-documented historical fact that the Iberian Peninsula was subjected to heavy deforestation during the High Middle Ages to allow for the grazing of Merino sheep, and thus for the development of the wool industry. This strong ecological disturbance could have precipitated the disappearance of *H. spontaneum.*

The occurrence of genotype CMb-2, CMe-2.1-2.2 in many Spanish and French barleys and *H. spontaneum* from Morocco (see Molina-Cano et al. 1987) suggests the introduction in the Iberian Peninsula of barleys that had already been domesticated in the Maghreb. This explanation could be possible on the basis of the historical records. Alternatively, the occurrence in the Iberian peninsula of wild barleys with that genotype in historical times cannot be ruled out and it might, thus, be formulated as an hypothesis.

The historical evidence

There are theories that have suggested that even in Greece cultivated barley was imported from the Fertile Crescent, possibly from the Anatolian Peninsula (see Renfrew 1989). However, other, ourselves included, have supported the idea of a possible domestication of barley in Europe (Dennell 1983; Barker 1985) or in Mediterranean regions, because wild barley remains tracing back to Paleolithic have been found in Greece (Hansen and Renfrew 1978).

The remains of cultivated barley have been found in two caves in Alicante (Mediterranean coast of Spain) that were dated with [C^{14}] to 4500 and 5590 B.C., respectively (R. Buxó personal communication), although the first historical records of Fenician and Greek visits to the Iberian Peninsula are dated at 800 B.C. (review in Tuñón de Lara et al. 1980). In fact a west Mediterranean Neolithic has been claimed (Tuñón de Lara et al. 1980), but imported from the east. In this context, two ways of putative penetration of the Neolithic wave, from the eastern Mediterranean, have been suggested: the most important one following the Mediterranean coast and a secondary one coming through central Europe via the Danubian basin. Iberia has been invaded on many occasions in historical times. In addition to the Fenicians and Greeks reported above, people from eastern and central Europe entered our peninsula at the beginning of the 5th century (review in Sayas Abengoechea and García-Moreno 1980), and Arabs coming from Morocco arrived at the beginning of the 8th century (review in Arié 1980). Any of these cultures could have brought barley with them from Greece, eastern and central Europe, and Morocco.

Conclusions

Despite the different possibilities emerging both from genetical and prehistorical research, we would like to postulate the following hypotheses as they seem to be in agreement with the evidence presented.

1) The origin of the Spanish two-rowed barleys could be explained by the following three processes, which are complementary to each other, in order of relative importance:

a) *In situ* domestication from an ancestral *H. spontaneum* that has disappeared,

b) Brought from the Maghreb by the Arabic invaders from the 8th century,

c) Brought from the eastern Mediterranean in Neolithic or historical times.

2) Barley would be, thus, a multicentric crop, domesticated along the Mediterranean basin and, perhaps, also in Tibet.

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