Theor Appl Genet (2005) 110: 613–619
DOI 10.1007/s00122-004-1878-3

ORIGINAL PAPER

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Chloroplast DNA microsatellite analysis supports a polyphyletic origin for barley

Received: 3 August 2004 / Accepted: 10 November 2004 / Published online: 26 January 2005

Abstract Five barley chloroplast DNA microsatellites (cpSSRs) were used to study genetic relationships among a set of 186 barley accessions—34 *Hordeum vulgare* ssp. *spontaneum* (HS accessions) from Morocco, Ethiopia, Cyprus, Crete, Libya, Iraq, Iran, Turkey, Afghanistan and Israel, 122 *H. vulgare* ssp. *vulgare* landraces (HV landraces) from Spain, Bolivia (old Spanish introductions), Morocco, Libya and Ethiopia and 20 modern European spring barleys (HV cultivars). All loci were polymorphic in the material studied, with the number of alleles per locus ranging from two to three. Fifteen multi-locus haplotypes were observed, 11 in HS accessions and seven in HV landraces and cultivars. Of the seven haplotypes found in the HV lines, three were shared with the HS accessions, and four were unique. Cluster analysis revealed two main groups, one consisting of HS accessions from Ethiopia and the HV landraces from Spain, Bolivia (old Spanish), Morocco and Ethiopia, whereas the other larger group contained all of the other accessions studied. Based on these grouping and the existence of haplotypes found in the HV landraces and cultivars but not in the HS wild barley, a polyphyletic origin is proposed for barley, with further centres of origin in Ethiopia and the Western Mediterranean.

Communicated by P. Langridge

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Introduction

The origin of cultivated barley has been considered to be monophyletic, stemming from a single domestication event around 8,000 years BC (Harlan and Zohary 1966). These authors defined *Hordeum vulgare* ssp. *spontaneum* as its wild ancestor and the Fertile Crescent, represented by an arch starting in Israel and ending in Iran, as the geographical region where domestication occurred. This is not, however, a consensus theory, as other evidence has suggested alternative patterns of domestication taking into account place and number of domestication events, such as Morocco (Molina-Cano et al. 1987), Ethiopia (Bekele 1983), Tibet (Xu 1982). In order to address these theories, we should first recognise that a centre of origin of a crop is the region where the wild ancestor and the domesticated form co-exist (De Candolle 1882, cited by Bakhteyev 1964). Vavilov (1926, cited by Harlan 1992) also added that the centre of origin should have the greatest genetic diversity of the crop. Although these concepts have evolved with time (for a discussion, see Harlan 1992), the principles continue to be relevant and broadly accepted by the scientific community.

Consequently, the discovery of *H. spontaneum* in places other than the Fertile Crescent and surrounding regions (review in Molina-Cano et al. 2002), such as Tibet, Morocco, Libya, Egypt, Crete and others, and the disclosure of the great genetic diversity of Ethiopian barleys have challenged this mono-centric theory of the origin of barley. With respect to the number of domestication events, the existence of two different brittle-rachis genes linked to eastern and western barleys (Takahashi 1955) has been argued as evidence of a polyphyletic origin (Zohary 1999), as is the extensive variation in phenotypic traits found in core and peripheral regions (Volis et al. 2000), the diversity in gene frequencies of esterases (Ladizinsky and Genizi 2001) and the high levels of single nucleotide polymorphisms (SNPs) present in wild and cultivated barleys

(Kanazin et al. 2002). Interestingly, the polymorphism found in a marker closely linked to the *vrs1* locus (Tanno et al. 2002) supports the existence of an independent domestication centre in the Western Mediterranean. Evidence for a polyphyletic origin, with domestication also in the Western Mediterranean, has also been proposed for olive tree (Besnard et al. 2002; Contento et al. 2002), grapevine in Italy (Grassi et al. 2003) and lentils in Greece (Halstead 1999). Most importantly, the absence of a wild ancestor in a given region today which may be considered as a centre of origin does not preclude the existence of the wild ancestor in ancient times when domestication occurred.

Since the discovery of weedy *H. spontaneum* populations in Morocco, we have been accumulating evidence to support a polyphyletic origin of barley (Molina-Cano and Conde 1980; Molina-Cano et al. 1982), and subsequent studies have shown genetic divergence with wild barleys from different regions (Molina-Cano et al. 1987, 1999). Our results have shown clear differences between the Moroccan and other *H. spontaneum* accessions based on nuclear restriction fragment length polymorphisms (RFLPs) (Molina-Cano et al. 1999). All of this evidence has led us to consider that barley has a polyphyletic origin and that its domestication occurred in regions other than the Fertile Crescent. The prehistorical evidence supporting our theory will be detailed in the Discussion section of the present paper although, unfortunately, this evidence is more sparse than what is presently available from the Fertile Crescent and Turkey, where archaeological expeditions have uncovered a large amount of materials since the beginning of the last century.

In contrast to our proposal of a polyphyletic origin of barley, Blattner and Madani Méndez (2001), Badr et al. (2000), Bothmer (personal communication) and Salamini et al. (2002) have argued against multiple origins of barley, suggesting that Moroccan *H. spontaneum* accessions are a result of introgression. The opinions of these latter investigators have been challenged, however, by Allanby and Brown (2003), who found the methods used to be inappropriate—i.e. anonymous band markers with neighbour-joining methods. In the study reported here, we used simple sequence repeat (SSR) markers to overcome these problems. Chloroplast (cp) microsatellites were used to elucidate species relationships within the genus *Hordeum* (Provan et al. 1999) and seven cpSSR loci were described, of which five were polymorphic in *H. spontaneum* accessions, with only one locus (hvcptrnLF) being polymorphic in the cultivated barley landraces.

In an attempt to unravel the origin for barley, we have sampled and assayed accessions of both cultivated and wild barleys from across the distribution range of the species, excluding the Far East, using cpDNA microsatellites (cpSSRs).

Materials and methods

Plant material

A total of 186 barley accessions—34 *Hordeum vulgare* ssp. *spontaneum* (HS) and 152 *H. vulgare* ssp. *vulgare* (HV)—were analysed (Table 1). Of the cultivated barleys, 132 were landraces (HV landraces) and 20 were

Table 1 Wild and cultivated barley germplasm studied

Country/Region	Type of material	Spike type	Number of accessions	Remarks
Morocco	Wild barley	Two-row	8	Own collection
Ethiopia	Wild barley	Two-row	2	USDA World Collection
Cyprus	Wild barley	Two-row	3	Cyprus Agricultural Research Institute
Crete	Wild barley	Two-row	1	Own collection
Iraq	Wild barley	Two-row	2	USDA World Collection
Libya	Wild barley	Two-row	2	Gatersleben Gene Bank
Iran	Wild barley	Two-row	4	USDA World Collection
Turkey	Wild barley	Two-row	3	USDA World Collection
Afghanistan	Wild barley	Two-row	5	USDA World Collection
Israel	Wild barley	Two-row	4	USDA World Collection
Spain	Cultivated barley landraces	Two- and six-row	45	Spanish Gene Bank
Bolivia/Spain	Cultivated barley landraces	Two- and six-row	10	Own collections and Braunschweig Gene Bank
Morocco	Cultivated barley landraces	Two- and six-row	37	USDA World Collection and own collection
Ethiopia	Cultivated barley landraces	Two- and six-row	30	USDA World Collection
Libya	Cultivated barley landraces	Six-row	10	USDA World Collection
Europe/USA	Barley cultivars	Two- and six-row	20	SCRI collection
Total			186	Thirty-four wild and 152 cultivated barleys

modern cultivars (HV cultivars). The origins of the HS barleys covered all of the known distribution range, except the Far East—i.e. Morocco, Libya, Crete, Cyprus, Ethiopia, Israel, Turkey, Iraq, Iran and Afghanistan. The landraces included two- and six-rowed, naked and hulled, *deficiens* (reduced size of the sterile spikelets) and normal types; these originated from Spain, Morocco, Libya and Ethiopia. Among the 55 Spanish accessions, 45 were from the Spanish Centre for Phytogenetic Resources (most of them collected before 1955), while ten were of the so-called *criolla* type, i.e. descendants from the barleys brought to South America by the Spanish conquerors in the sixteenth century. Of the HV landraces, two were two-rowed *deficiens* types, collected in the Bolivian Andes (collected by G. Arias), and the remaining were six-rowed types obtained from the Braunschweig Gene Bank (Germany).

The 20 European cultivars (HV cultivars) were chosen from a large array (more than 300 entries) as a representative sample of the genetic variability existing within the cultivated spring barley gene pool. (J.R. Russell, in preparation).

cpDNA microsatellite polymorphism

Seeds were germinated and DNA extracted using Qiagen Dneasy Mini Extraction kit as per the manufacturer's instructions (Qiagen, Valencia, Calif.). Primer sequence information is given in Table 2. PCR analyses were carried out in a total volume of 10 µl with 1× PCR buffer containing 1.5 m *M* $MgCl_2$, 1 U *Taq* polymerase (Roche, Indianapolis, Ind.), 200 μM dNTPs, 10 pmol [^{33}P]-ATP end-labelled forward primer (Sambrook et al. 1989), 10 pmol reverse primer and 50 ng genomic DNA. Touchdown-PCR was performed on a PE 9700 (Applied Biosystems, Foster City, Calif.) as follows: one cycle of 5 min at 94°C; one cycle of 30 s at 94°C, 30 s at 65°C and 30 s at 72°C; seven cycles where the annealing temperature was reduced by 1°C per cycle, followed by 24 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; a final 7-min cycle at 72°C. The products were resolved on 6% denaturing polyacrylamide gels, which were dried and exposed to X-ray for 24 h.

Statistical analyses

Since there is no recombination in the chloroplast genome, allele sizes were combined to generate multi-locus haplotypes. We used cluster analysis to assess the overall genetic similarity among haplotypes, applying Ward's method based on the CITY-BLOCK distance matrix (Sneath and Sokal 1973). STATGRAPHICS package, ver. 4.1 (Statistical Graphics Corporation 1999), was used to generate a dendrogram based on the similarity matrix and a principal component analysis three-dimensional graph. The Ward's clustering starts out with t separate operational taxonomic units (OUTs), grouping them successively into $t-1$, $t-2$, $t-3$,..., 1 taxa and computing at each stage an objective function (the within-group sum of squares) that measures the desirability of the particular arrangement of the t OUTs into $k < t$ taxa at any one stage. The CITY-BLOCK distance, also called the MANHATTAN distance, was computed as:

$$d(j,k) = \sum_{i=1}^{n} \left| X_{ij} - X_{ik} \right|$$

where $d(j, k)$ stands for the distance between haplotypes j and k using barley accessions 1 to n. Nei's (1973) diversity index was used, computed as:

$$H_T = 1 - \sum P_i^2$$

in which P_i is the frequency of the ith SSR allele.

For a comparison of methodology, cluster analysis, using the proportion of shared alleles and absolute distance measures, were generated with MICROSAT (Minch et al. 1997) and the NEIGHBOR programme of the PHYLIP package (Felsenstein 1993).

Results

The analysis of chloroplast microsatellites are summarised in Tables 2 and 3. All five loci analysed were polymorphic in the material studied (Table 3), and the number of alleles per locus ranged from two to three, with diversity values ranging from 0.032 (locus

Table 2 Description of the primers used, number of alleles and diversity values (Nei 1973) (T_m annealing temperature)

Locus	Repeat	Location	Primers (5′–3′)	T_m (°C)	Size (bp) (range)	Number of alleles	Diversity values
hvcppsbK	$(A)_{11}$	*psbK*/ ORF174 intergenic region	TAGCCTTTGTTTGGCAAGCT TAAAACTTCTCGGCTTTTACCC	60	121–122	2	0.084
hvcppsbA	$(T)_8$	Downstream of *psbA*	AATGGATAAGGTTTTTCTGC TGAATAGAAAGATTAAGAAGA	60	145–146	2	0.032
hvcptrnS1	$(A)_7$ CGC $(T)_{11}$	Downstream of *trnS*	CTTTAGCGGGCATTTCCATAA TGGTGGATTTGATAAGAACCC	60	126–135	3	0.322
hvcptrnS2	$(T)_{11}$	Downstream of *trnS*	CAACTCCTTTGCGCTACACAAC CCCTTTTTTCCCATTCC	60	112–114	3	0.355
hvcptrnLF	$(C)_9$	*trnL*/*trnF* intergenic	GAGTATCGGCAAGAAATCTTG GTCAAAATTTGAAAGGGGGG	60	100–102	3	0.472

Table 3 Haplotypes defined and size (in basepairs) of five cpDNA microsatellites of wild and cultivated barleys

Haplotype	hvcppsbK	hvcppsbA	hvcpptrnS1	hvcpptrnS2	hvcpptrnLF	Remarks
1	121	146	135	114	102	1 *Hordeum spontaneum* from Libya
2*	122	146	135	113	102	4 European cultivars
3	122	146	135	114	101	1 *Hordeum spontaneum* from Afghanistan
4*	121	146	135	113	101	4 Spanish and 1 Moroccan cultivated barley landraces
5	122	146	135	113	101	38 Spanish, 8 old Spanish *criolla*, 32 Moroccan, 9 Libyan and 5 Ethiopian cultivated barley landraces; 9 European and 2 North American barley cultivars; 8 *Hordeum spontaneum* from Morocco
6	122	146	135	114	100	2 *Hordeum spontaneum*, from Iran and Israel
7	122	146	127	114	101	1 *Hordeum spontaneum* from Crete
8	121	146	135	113	100	2 *Hordeum spontaneum* from Afghanistan
9	122	146	127	113	101	1 *Hordeum spontaneum* from Libya
10	122	146	135	113	100	1 Moroccan cultivated barley landrace, 4 European cultivars, 15 *Hordeum spontaneum*, from Palestine, Turkey, Cyprus, Afghanistan and Iran.
11*	122	146	135	112	100	1 cultivated barley landrace from Libya
12*	122	146	127	112	100	4 Ethiopian, 1 Moroccan and 1 old Spanish *criolla* cultivated barley landraces
13	122	146	126	112	100	21 Ethiopian, 2 Moroccan and 1 old Spanish *criolla* cultivated barley landraces; 1 *Hordeum spontaneum* from Ethiopia
14	122	145	135	114	100	2 *Hordeum spontaneum* from Iraq
15	122	145	126	112	100	1 *Hordeum spontaneum* from Ethiopia

* Haplotypes present in cultivated barley but not in *Hordeum spontaneum*.

hvcppsbA) to 0.472 (locus hvcptrnLF) (Table 2). Fifteen multi-locus haplotypes were identified (Table 3), of which 11 were present in the HS lines and seven in the HV landraces and cultivars.

Of the seven haplotypes found in cultivated barley, three were observed in the HS lines and the remaining four were unique to HV lines. These latter four haplotypes, which are not found in wild barley, are represented by haplotype 2 in four modern European cultivars, by haplotype 4, found in four Spanish and one Moroccan landraces, by haplotype 11, in one Libyan landrace and by haplotype 12, in a Moroccan, a *criolla* (old Spanish) and four Ethiopian landraces. Interestingly, of the six accessions possessing haplotype 12, four are of the *deficiens* type (the *criolla*, the Moroccan, and two of the four Ethiopian landraces) and the other two accessions, both Ethiopian lines, are so far undetermined.

An analysis of genetic similarity among haplotypes is presented in Fig. 1. Two main clusters are observed, clusters A and B, with cluster B being further subdivided into two sub-clusters, C and D. Cluster A consists of HS lines from Ethiopia (haplotypes 13 and 15) and HV landraces from Ethiopia, Spain, old Spanish and Morocco (haplotypes 12 and 13). Haplotype 13 is shared by the HS and HV landraces from Ethiopia, but haplotype 12 has not been found in any of the HS accessions studied. Cluster B contains no Ethiopian HS lines, but all of the other origins of HS wild barley studied in this project are represented in this cluster. The larger of the sub-clusters, C, consists of haplotype 5, the most common haplotype and found in HV landraces from Spain, old Spanish (*criollas*), Morocco, Ethiopia and Libya, in modern European and North American cultivars and in all of the accessions of HS from Morocco. Libya, Afghanistan, Crete, (haplotypes 3, 7 and 9), Iran, Iraq, Israel, Turkey, Cyprus, Afghanistan, (haplotypes 6, 10 and 14) and European cultivars (haplotype 2), one Moroccan landrace and four European cultivars (haplotype 10) are also included in sub-cluster C. Sub-cluster D contains HS from Libya (haplotype 1), Afghanistan (haplotype 8) and cultivated landraces from Spain and Morocco (haplotype 4).

We compared the distribution of haplotypes with alleles at each of the five cpSSR loci using cluster analysis on matrices generated with different measures of similarity, i.e. CITY-BLOCK distance, proportion of shared alleles and absolute distance (data not presented). In all three cases, haplotypes 12, 13 and 15 clustered, representing Ethiopian HS and landrace lines and a selection of Moroccan and Spanish landraces. Similarly, haplotypes 4 and 8, 7 and 9, and 6 and 14 always clustered. In the case of CITY BLOCK and the proportion of shared alleles, the clusters were separated on the basis of the hvcppsbK and hvcpptrnS1 locus (121 bp/122 bp and 126 bp or 127 bp/135 bp, respectively). Further evidence of clustering between Ethiopian landraces and HS lines is shown by the shared haplotype data, with the most frequent haplotype being shared by both landraces and HS lines; a similar result was observed with Moroccan landraces and HS accessions.

Discussion

Our chloroplast-based cluster analysis supports multiple domestication events in barley. The presence of two main clusters suggests different domestication centres—Ethiopia (cluster A) and a composite centre consisting of a wide geographic origin of accessions (cluster B). Cluster B comprises HS lines from the Fertile Crescent, a known site of domestication (haplotypes 5 and 10) as well as HV cultivars and landrace lines that have no corresponding HS haplotype (haplotypes 2, 4 and 11). The same is true for haplotype 12 of cluster A,

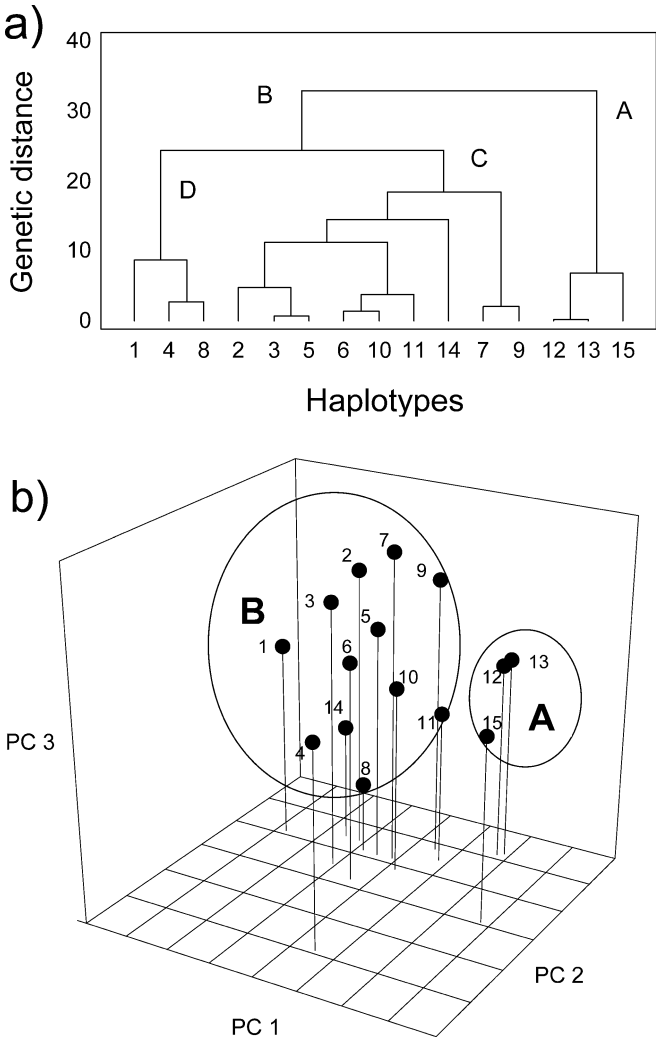

Fig. 1 **a** Dendrogram calculated using Ward's clustering on the CITY-BLOCK distance matrix of the 15 cpDNA microsatellite haplotypes found in a collection of 34 *Hordeum spontaneum* accessions and 152 cultivated barleys, including landraces and modern cultivars, after being characterised for five cpSSR loci. The composition of each haplotype group is as presented in Table 2. **b** A principal component analysis three-dimensional graph accounting for 78.9% of the total variance. Note the good correspondence between both analyses with respect to the grouping of haplotypes, particularly clusters A and B. The location of haplotypes 1 and 4 in (**b**) reflects more reliably its distance from the other members of cluster B (see Discussion)

whose wild ancestor is not present within the accessions in this study. Unpublished information on a cpSSR analysis of 347 *H. spontaneum* lines (J.R. Russell et al., in preparation) does show that haplotype 2 is found in Libyan *H. spontaneum*. However, there is no known *H. spontaneum* (HS) accession with haplotype 4 in the present study, suggesting that the ancestral source could have been a *H. spontaneum* genotype, either from Morocco or Spain, that no longer exists. It is important to point out that haplotype 5, which we have found only in *H. spontaneum* from Morocco, has also been observed in HS wild barleys from the Fertile Crescent and Turkmenistan, but at a relatively low frequency of 0.067 (J.R. Russell et al., in preparation). Haplotype 11, which we have found in a cultivated landrace from Libya, has also been found in wild accessions from Israel and Jordan (J.R. Russell et al., in preparation), therefore this cultivated accession could have been derived from an ancestral *H. spontaneum* from the Fertile Crescent.

Our results (Table 3, Fig. 1) show that HV landraces and HS lines from the same geographical origin share the same haplotype and that HV landraces with unique haplotypes were not present in any of the HS lines surveyed in our investigation. We can hypothesise that there have been several domestication events in the following regions: (1) the well-recognised Fertile Crescent region (sub-cluster C); (2) Ethiopia (cluster A); (3) the Western Mediterranean (haplotype 4). The idea of Ethiopia as a centre of origin of barley has been around since the time of Vavilov, partly because of the vast phenotypic diversity of landrace barleys found in this region. However, the absence of wild barley led Vavilov to reject his initial theory (review in Molina-Cano et al. 2002), although Bekele (1983) later found further evidence to suggest Ethiopia as a possible centre of origin. The USDA World Barley Collection has two accessions of *H. spontaneum* from Ethiopia (H. Bockelman, personal communication) which were used in this study, and the genetic distance between the Ethiopian accessions and those in cluster B are clear proof of genetic divergence prior to domestication (the alleles differ at two or three of the five loci for the Ethiopian HS lines (haplotypes 13, 15) and the Fertile Crescent HS lines (haplotypes 14, 6)

We have postulated a centre of origin of barley in the Western Mediterranean since discovering *H. spontaneum* in Morocco 20 years ago (Molina-Cano et al. 1987). Our recent RFLP study using a wide array of wild barley accessions (Molina-Cano et al. 1999) demonstrated the large genetic distance between the Moroccan accessions and other wild barley lines. Our earlier findings (Molina-Cano et al. 1987) were, however, disputed on the grounds of the introgressed origin of the Moroccan HS barley accessions (Badr et al. 2000; Giles and Leftkovitch 1984, 1985) Although, the hybrid nature of our material has been recognised (Molina-Cano et al. 1982) as a weed thriving in cultivated six-rowed barley fields, this present study clearly shows that the Moroccan HS accessions share the uni-parentally inherited cytoplasm type of the prevailing Moroccan cultivated barley landraces. Such a result would be expected if the Moroccan cultivated lines would have been domesticated from Moroccan HS lines with the nuclear genome of the Moroccan weed barley, (Molina-Cano et al. 1999), which, in turn, is a relic from the ancestral genuinely wild *H. spontaneum* populations that could have existed there before the drying out of the Sahara from the third millennium BC onwards (Harlan 1992).

Although we do not know how to relate the centres of Ethiopia and the Western Mediterranean in order to establish which is subordinate, there is some pre-his-

torical rationale linking both regions. In the southern Iberian Peninsula, archaeological remains dating from the end of the fourth millennium BC have been found recently that differ greatly from those pre-dating this period. There are differences not only in pottery and the stone industry but also in relation to the economy, to the way people established settlements and in symbolic expression. These matters also relate to art, religion and to funeral rites. It appears unlikely that such profound changes can be explained by a rapid evolution in this area. A more plausible explanation is the movement of various groups of people into the region (Escacena 2000). This new evidence indicates strong similarities with the cultural model of the Pre-dynastic Egyptian world, especially with the Badarian period (Arkell 1975). If we consider the remains originated from the Sahara (Childe 1976; Clark 1982; Drioton y Vandier 1981; Lhote 1975), it can be assumed that the new Hispanic culture shares the same origins (Escacena et al. 1988). As a consequence of the drying out of the environment, different pastoral communities would have begun to disperse progressively to the peripheries of the desert (Lhote 1965; Mozzolini 1995), thereby generating similar cultural effects as to those that occurred at the Lower Nile in the western Maghrib and the southern Iberian Peninsula (Cohen 1981). At the same time, the Saharan groups would have become Neolithic some millennia ago, starting with the expansion of farming practices from Ethiopia, Somalia and Sudan (Hugot 1974; Lhote 1975).

Taking into account all of the evidence, we propose a polyphyletic origin for barley, as has been suggested for other species in the region, such as lentil (Halstead 1999), olive tree (Besnard et al. 2002; Contento et al. 2002) and cattle (Wendorf and Schild 1998; Hanotte et al. 2002), which were domesticated earlier and/or independently in the Fertile Crescent (Bradley et al. 1996). However, further research is required with new collection missions to Ethiopia, Morocco and Libya to gather new samples of *H. spontaneum*, which could allow us to further elucidate the origin of barley.

Acknowledgements The Spanish group is funded by INIA and CICYT. J.R. Russell and W. Powell are funded by the Scottish Executive Environment and Rural Affairs Department (SEERAD).

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