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J. L. Molina-Cano · M. Moralejo
E. Igartua · I. Romagosa

Further evidence supporting Morocco as a centre of origin of barley

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Abstract Thirty-five populations of *H. spontaneum* from nine countries, encompassing almost all the known range of distribution of the species, Afghanistan, Crete (Greece), Cyprus, Iran, Iraq, Israel, Libya, Morocco and Turkey, were studied utilizing RFLP markers (21 probes with three restriction enzymes) distributed across all seven barley chromosomes in an attempt to unveil the genetic dissimilarities existing among them. UPGMA clustering, based on the Nei and Li (1979) similarity coefficient, produced a dendrogram where three clusters could be defined: two with a clear geographical distinction (Morocco and Cyprus) and another one grouping all the Asian/Middle Eastern populations, except for an accession from Iran that clustered separately. These results confirm our previous work and suggest that barley domestication could also have taken place outside the Fertile Crescent, particularly in Morocco.

Key words Barley · Domestication · *Hordeum spontaneum*

Introduction

The centre of origin of a crop may be defined as the region where domestication first took place and where the wild ancestor and the derived cultivated species co-exist. The most widely accepted hypothesis on the origin of cultivated barley defines the Fertile Crescent as its centre of origin (reviewed by Harlan 1992). One of the main factors supporting this hypothesis is the presence in that region of *Hordeum spontaneum* C. Koch, the nearest wild ancestor of cultivated barley. The occurrence of this wild barley also in southern Morocco (Molina-Cano and Conde 1980) changed the prevailing view on the distribution range of the species, previously thought to have its western limit in Libya. The true *H. spontaneum* nature of those wild barley plants collected in Morocco was confirmed at the Vavilov Institute (F. Bakhteyev, personal communication). This preliminary finding led to a second expedition where more populations were found, some of which were subsequently included in a collection (Molina-Cano et al. 1982). Subsequent work has further challenged the concept of a unique centre of origin, widening the area to both the west and east, from Morocco to Tibet (Molina-Cano et al. 1987; Moralejo et al. 1994; Xu 1982; Ma et al. 1987).

Early genetic studies revealing the differences between the Moroccan wild barley populations and those from the Fertile Crescent were carried out using both morphological characters and CM proteins, a group of endosperm proteins that are soluble in chloroform-methanol mixtures (Salcedo et al. 1982), as markers. The results showed clear differences between the two groups of accessions and led to the proposition of a possible multicentric origin for barley, which could have been domesticated at any point on the long arch extending from the Southern edge of the Mediterranean basin, encompassing the Fertile Crescent and ending in the Tibet.

The development of molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs) etc. allowed a reassessment of the genetic diversity and phylogenetic relationships of *Hordeum* species; these had previously been based on studies of morphological, isozyme and protein markers. As for many other

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J. L. Molina-Cano (✉) · M. Moralejo · I. Romagosa
Centre UdL-IRTA. Av Alcalde Rovira Roure 177. 25178 Lleida, Spain
E-mail: joseluis.molina@irta.es

E. Igartua
CSIC, Estación Experimental de Aula Dei, Montañana 177, Zaragoza, Spain

species, studies with molecular markers have produced results highly consistent with previous knowledge and have proved these markers to be the best tools currently available to examine the genetic relationships of germplasm pools. Examples of such studies include the following: Zhang et al. (1993) assessed the genetic diversity existing between 25 populations of *H. spontaneum* from Israel with isozyme and RFLP markers; Petersen et al. (1994) used RFLPs to analyse the genetic diversity among wild barleys and cultivated forms from Israel, Iran and Turkey; Saghai-Maroof et al. (1995) studied molecular diversity between cultivated and wild barley with RFLPs; Melchinger et al. (1994) and Casas et al. (1998) successfully used RFLPs to classify European cultivars of barley; Bjørnstad et al. (1997) studied Ethiopian barley landraces and a *H. spontaneum* accession from Afghanistan. Other markers used included RAPDs (Weining and Henry 1995; Baum et al. 1997), amplified fragment length polymorphisms (AFLPs) (Pakniyat et al. 1996) and rDNA variability (Chalmers et al. 1992). Genetic markers such as AFLPs have proved very useful in identifying a population of *Triticum monoccocum* ssp. *boeoticum* from the Karacadag mountains in Turkey as the likely wild progenitor of einkorn wheat (*Triticum monoccocum* ssp. *monoccocum*) (Heun et al. 1997).

We decided to use RFLP markers to reassess the genetic similarity between Moroccan and non-Moroccan accessions of wild barley in an attempt to shed more light on the origin of the cultivated forms. The choice of RFLPs was based on their excellent reliability (Karp et al. 1997) and on the possibility of sampling all seven barley chromosomes by choosing appropriate probes of a known location.

Materials and Methods

Plant material

The material used, listed in Table 1, consisted of 35 populations of *Hordeum spontaneum* from nine countries, thereby encompassing almost all the known range of distribution of the species except the far eastern region (Tibet). The number of entries per country of origin ranged from 8 from Morocco to single entries from Crete (Greece) and Libya, and depended on their availability. The populations were represented as a bulk of individuals, i.e. of pure lines, because our aim was not to study the intra-population variability but the overall genetic diversity existing between populations and, ultimately, between countries of origin.

RFLP analysis

RFLP probes

Twenty-one probes were chosen in order to obtain a fairly uniform coverage of the barley genome. The probes were, for chromosome 1H, Amy2 and RisP103; chromosome 2H, MWG858 and ABG008; chromosome 3H, ABG471, ABG315 and ABC174; chromosome 4H, Bmy1, MWG611, MWG058, ABG366 and ABG472; chromosome 5H, BCD98 and MWG706; chromosome 6H, MWG620; chromosome 7H, CDO749, ABC302, ABG390, ABG463, ABC309 and WG364. Clones ABC, ABG, Amy2 and Bmy1 were kindly supplied by A. Kleinhofs (Washington State University, Pullman, Wash.); MWG by A. Graner (Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany); RisP103 by H. Giese (Risø National Laboratory, Roskilde, Denmark), and CDO and WG by M. Sorrells (Cornell University, Ithaca, N.Y.).

DNA preparations

Twenty seeds of each population (accession) were sown, and seedlings were grown in a greenhouse for 15 days. Barley seedlings were harvested, frozen in liquid nitrogen and stored at -80°C. DNA was extracted, bulking the material from all seedlings per accession, using a CTAB procedure described by Saghai-Maroof et al. (1984).

Southern blotting

DNA (15 g) was digested with the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII (4 units/g DNA) according to the manufacturer's instructions (BRL). Horizontal gel electrophoresis was performed on 0.8% agarose gel at 20 V overnight. DNA was then transferred to a Hybond N^+ membrane (Amersham) under alkaline conditions (1.5 *M* NaCl, 0.4 *M* NaOH).

Hybridization methods

Inserts were purified by GeneClean (Bio 101) after digestion of the original plasmid. Probes were labelled (50–100 ng) with [^{32}P]-dCTP by random priming using Ready to Go kit (Pharmacia). The blotted membranes were prehybridized at 65°C for 1 h in Rapid-hyb buffer (Amersham), and the hybridization was performed overnight using an identical buffer and temperature. The hybridized membranes were washed twice for 15 min in 2 × SSC, 0.1% SDS at room temperature and twice for 15 min in 0.2 × SSC, 0.1% SDS at 65°C, then exposed to X-ray films at -80°C with an intensifying screen for 2–7 days.

Distance measurements and statistical analysis

Genetic distance (*GD*) between 2 accessions was calculated as one minus the similarity index (*GS*) of Nei and Li (1979):

$$GS_{ij} = 2N_{ij}/(N_i + N_j),$$

$$GD_{ij} = 1 - GS_{ij},$$

where *Nij* is the number of bands in common between accessions *i* and *j*, and *Ni* and *Nj* are the numbers of bands in the respective cultivars, with all clone-enzyme combinations (CECs) considered. Cluster analysis (UPGMA) of the similarity matrix was used to reveal associations among the accessions. Genetic distances and cluster analysis were performed using appropriate procedures of the computer package NTSYS-PC (Rohlf 1989). Significance of pooled genetic distances among countries was tested against genetic distances within countries by means of a bootstrap procedure.

Table 1 Origins of the accessions of *H. spontaneum* studied

Origin	Number	Code	Site	Seed source	Sender
Morocco (MO)	1	HS1	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	2	HS2	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	3	HS3	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	4	HS4	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	5	HS5	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	6	HS6	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	7	HS7	Djebel Siroua Range[a]	Own collection[b]	–
Morocco (MO)	8	HS8	Djebel Siroua Range[a]	Own collection[b]	–
Crete (CR)	1	HS20	Unknown	Univ Polit, Madrid[c]	C. Gómez Campo
Cyprus (CY)	1	87-226	Akhera	Agric Res Inst, Nicosia	A. Hadjidichristodoulou
Cyprus (CY)	2	87-341	Athalassa	Agric Res Inst, Nicosia	A. Hadjidichristodoulou
Cyprus (CY)	3	Bulk	Akhera	Agric Res Inst, Nicosia	A. Hadjidichristodoulou
Afghanistan (AF)	1	PI 219921	Kandahar	USDA[c]	J.C. Craddock
Afghanistan (AF)	2	PI 220523	Faryab	USDA	H.E. Bockelman
Afghanistan (AF)	3	PI 220664	Herat	USDA	H.E. Bockelman
Afghanistan (AF)	4	PI 212305	Jowzjan	USDA	H.E. Bockelman
Afghanistan (AF)	5	PI 366431	Farah	USDA	H.E. Bockelman
Iraq (IQ)	1	PI 219796	Salahuddin	USDA[c]	J.C. Craddock
Iraq (IQ)	2	PI 254894	Arbat	USDA[c]	J.C. Craddock
Iran (IR)	1	PI 227019	Khuzestan	USDA	H.E. Bockelman
Iran (IR)	2	PI 401367	Khuzestan	USDA	H.E. Bockelman
Iran (IR)	3	PI 466606	Unknown	USDA	H.E. Bockelman
Iran (IR)	4	PI 466627	Unknown	USDA	H.E. Bockelman
Iran (IR)	5	PI 466634	Unknown	USDA	H.E. Bockelman
Israel (IS)	1	PI 282597	Unknown	USDA	H.E. Bockelman
Israel (IS)	2	PI 282615	Unknown	USDA	H.E. Bockelman
Israel (IS)	3	PI 466577	Herzliyya	USDA	H.E. Bockelman
Israel (IS)	4	PI 466528	Bet She'an	USDA	H.E. Bockelman
Israel (IS)	5	PI 466322	'Atlit'	USDA	H.E. Bockelman
Israel (IS)	6	PI 405238	Unknown	USDA	H.E. Bockelman
Turkey (TU)	1	PI 245739	Urfa	USDA	H.E. Bockelman
Turkey (TU)	2	PI 466664	Unknown	USDA	H.E. Bockelman
Turkey (TU)	3	PI 245742	Urfa	USDA	H.E. Bockelman
Turkey (TU)	4	PI 596285	Mardin	USDA	H.E. Bockelman
Libya (LI)	1	AHOR 9721/82	Cyrenaica	Gatersleben	K. Hammer

[a] A land strip of no more than 70×30 km in the hillsides of the Djebel Siroua mountain range, located south of Morocco between the Great Atlas and the Anti-Atlas mountains
[b] Molina-Cano et al. (1987)
[c] Accessions conserved by Molina-Cano, formerly at La Cruz del Campo (Seville, Spain) and then at IRTA (Lleida, Spain), as bulk populations

Results and discussion

RFLP variation

Of the 63 clone-enzyme combinations (CEC) possible, only 52 could either be performed, or proved clearly readable. Forty-eight (92.3%) of the 52 CECs showed polymorphic RFLP patterns, with 10 monomorphic and 206 polymorphic bands. Five polymorphic CECs presented single-band patterns (only 1 band per accession), with an average of 3.6 bands per CEC, whereas 43 CECs presented multiple-band patterns (more than 1 band in at least 1 accession), with an average of 4.6 bands per CEC. Overall, there was an average of 1.35 bands per accession for each polymorphic CEC.

Genetic diversity could not be calculated following classical polymorphic indices as bands could not be unequivocally assigned to alleles. The reason for this was that the accessions had been maintained as bulk populations and hence possibly comprised genetically distinct types. DNA extraction was done in bulk for a group of about 20 seedlings per accession. Thus, it is possible that we have detected more than one allele per locus and accession in some cases, if at least two alleles were abundant enough in an accession (population). Michelmore et al. (1991) tested the sensitivity of RFLP and RAPD techniques to detect the rarer allele in a mixture of two genotypes (actually, two different *Lactuca* species). The rarer allele was consistently detected in mixtures where its proportion was above 0.1. If that estimate of the sensitivity threshold is roughly correct then, in the present study, we might be able to detect most moderately abundant alleles for all populations (accessions). Only the truly rare alleles (approximately $P < 0.1$) would be undetectable.

Cluster analysis and geographical grouping

The cluster analysis performed on the similarity matrix produced the dendrogram presented in Fig. 1. Four main groups can be distinguished: a large cluster comprising most of the accessions of Asian/Middle Eastern origin, two compact clusters which correspond exactly to Morocco and Cyprus and a cluster formed by just 1 accession from Iran (code Iran4). The accession Iran4 is the most distinct of all (its average distance from all other accessions is the largest of all, 0.44), even though it has no unique bands. Its outermost position in the tree is due to the large distances that it presents from all accessions from Israel and Turkey, Morocco and Cyprus. Consequently, it does not fit into any of the more numerous groups because it is almost equidistant from them all. The cluster comprising the other Asian accessions contains three approximate groupings, although they are composed of populations from different countries. Baum et al. (1997) also found little difference among countries when studying *H. spontaneum* accessions from Iran, Israel and Turkey. It seems that the only geographic origins with clearly distinct genetic constitutions were Cyprus and Morocco. This is supported by the data presented in Table 2, which gives the average distances among origins. The only significant distances among pairs of origins involved either Cyprus or Morocco.

The average number of bands detected per accession (Table 2) varied between 60 and 72 (for Cyprus, which was the most polymorphic origin). These figures were not much larger than the 61 bands detected in two inbred line cultivars also included in the experiment (data not presented). The low band numbers per accession detected may be caused by either (or both) of two causes: a low intrapopulation polymorphism, or a low power of resolution of the specific RFLP procedures used in this study, which would enable the detection of only the more abundant alleles.

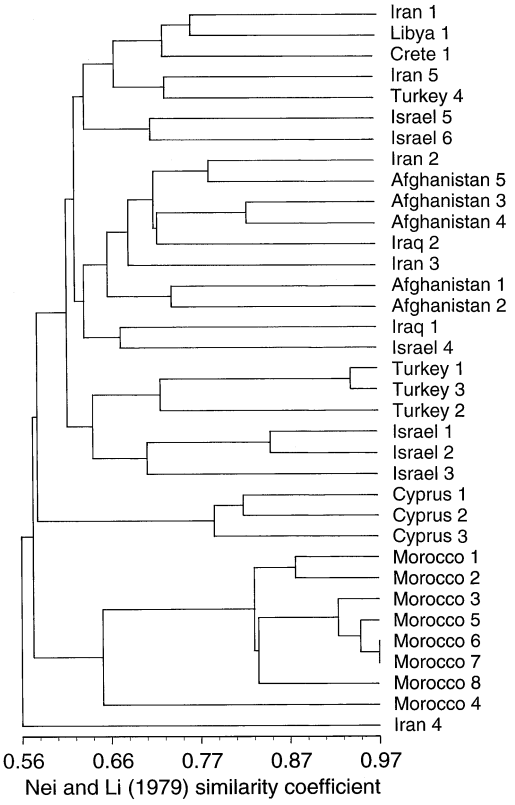

Fig. 1 Dendrogram showing the grouping of 35 *H. spontaneum* accessions based on cluster analysis (UPGMA) of RFLP variation among them. Accessions are labelled according to the "Origin" and "Number" columns in Table 1

The number of unique bands per origin was also calculated (Table 2). This figure represents the percentage of bands present in 1, or several, accessions from one origin which do not occur in any other origin. Israel and Cyprus presented the largest percentages of unique bands.

Table 2 Genetic distances (Nei and Li 1979) between countries, calculated by averaging distances among pairs of accessions for each pair of countries. Average genetic distances within countries are given along the diagonal

Origin	n[a]	Bands per accession[b]	Unique bands (%)[c]	All other origins	AF[d]	CR	CY	IR	IQ	IS	LI	MO	TU
Afghanistan	5	64.6	3.6	0.38	*0.28*								
Crete	1	64.0	4.7	0.39	0.40	–							
Cyprus	3	72.0	9.9	0.43**	0.41	0.44	*0.21*						
Iran	5	65.6	4.7	0.39	0.34	0.33	0.44**	*0.34*					
Iraq	2	63.0	0.0	0.39	0.33	0.41	0.39	0.37	*0.34*				
Israel	6	65.7	10.1	0.41**	0.38	0.40	0.43**	0.40	0.38	*0.36*			
Libya	1	60.0	0.0	0.37	0.39	0.26	0.40	0.35	0.41	0.36	–		
Morocco	8	62.8	4.0	0.42**	0.40	0.41	0.46**	0.42**	0.43**	0.44**	0.39	*0.19*	
Turkey	4	64.5	4.8	0.39	0.38	0.36	0.42*	0.38	0.40	0.37	0.34	0.42*	*0.31*

*,** Significantly larger than pooled distance within countries for $P < 0.05$ and $P < 0.01$, respectively
[a] Number of accessions per origin
[b] Average total number of bands per accession of each origin for the 48 polymorphic CECs
[c] Average percentage of bands present in either accession of one specific origin and absent in all other origins
[d] For abbreviations, see Table 1

A global consideration of all the results presented above gives the following picture of diversity distribution in the set of accessions studied, even although sample size within origins may be too small to draw definitive conclusions: the accessions from Cyprus and Morocco form two distinct, geographically very compact groups. The diversity within these two groups was low, as indicated by the low average genetic distances (0.21 and 0.19), in Table 2. The entries from Cyprus presented the rarest combinations of bands (large percentage of unique bands), whereas Moroccan entries showed low polymorphism with mostly commonly found bands.

The Moroccan cluster (Fig. 1) showed the highest level of internal similarity and contained the most closely related pair of accessions (Morocco6 and 7). The least similar population within this cluster was Morocco4 (similarity coefficient 0.66). This can be explained because this accession possesses a tough rachis, similar to cultivated barley, suggesting introgression from cultivated forms. This is not surprising, since the Moroccan *H. spontaneum* survives as a weed in fields of cultivated barley (Molina-Cano et al. 1982). The similarity existing among the Moroccan accessions can be explained by the size of the geographical area where they were collected – no more than 70×30 km, on the hillsides of the Djebel Siroua mountain range, located in the south of Morocco between the Great Atlas and the Anti-Atlas mountains – and the small number of individuals constituting each weedy population. Conversely, the populations from the Middle East are comprised of many more individuals. Of more than 25 Moroccan populations collected (Molina-Cano et al. 1982) only 8 were able to germinate. Three more populations were found during the same collecting trip but in the Middle Atlas mountains, located 400 km to the north. Unfortunately, this material was immature when collected and did not germinate subsequently. We can tentatively conclude, since no other collection of Moroccan wild barleys has been reported in the literature, that our 8 populations are probably only a small sample of existing Moroccan wild barleys.

Consistent geographical patterns within the Asian cluster have not been found (Fig. 1), confirming the observations of other workers studying *H. spontaneum* from this region. Baum et al. (1997) found no differences attributable to countries for 20 populations from Israel, Iran and Turkey. Weining and Henry (1995) studied 50 populations from Israel, Turkey, Iran, Pakistan and of unknown origin and did not find any geographical pattern among the groups formed. Turuspekov et al. (1996) studied 27 populations from Turkmenistan and found a balanced distribution of the variation among and within geographical origins. By contrast, Pakniyat et al. (1996) found geographical grouping patterns when studying 39 populations, each representing by a single genotype, from the Fertile Crescent arch. The monogenotypic composition of each accession and the type of marker used (AFLPs) could probably explain this result.

Phylogenetic conclusions

The results presented above and our previous work (Molina-Cano et al. 1987; Moralejo et al. 1994) have demonstrated clear-cut genetic differences between the Moroccan populations of *H. spontaneum* and those of other origins within the distribution range of the species. These genetic differences were always consistent, regardless of the different nature of markers used: morphological, CM proteins and, currently, RFLPs.

On the other hand, the *H. spontaneum* material from Tibet analysed by Ma et al. (1987) turned out to be very different from that from Israel, particularly with regard to spike colour, esterase isozymes, habit type and rachilla hairs, and these workers supported the hypothesis of Tibet as another centre of origin of cultivated barley, distinct from the Fertile Crescent.

Our results gathered over the last 15 years (Molina-Cano and Conde 1980; Molina-Cano et al. 1982, 1987; Moralejo et al. 1994) and particularly the ones presented here, together with those from Chinese workers (Xu 1982; Ma et al. 1987), confirm the presence of *H. spontaneum* in regions very distant from the classically accepted range of distribution of the species. The Moroccan and Tibetan populations of *H. spontaneum* have proved to be genetically distinct from those from the Fertile Crescent. As the presence of the immediate wild ancestor is one of the main supports for proposing this last region as the centre of origin for cultivated barley, we suggest the hypothesis of a multicentric origin for cultivated barleys that may have been domesticated within a great 'arch' starting in Morocco and ending in Tibet.

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