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RAPD variation in wild populations of four species of the genus *Hordeum (Poaceae)*

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Abstract The genetic variation of 102 natural populations of wild barley growing in Spain was assessed using RAPDs (random amplified polymorphic DNA). The plant material included the annual species *H. marinum* subsp. *marinum* (22 populations) and subsp. *gussoneanum* (14), *H. murinum* subsp. *murinum* (7) and subsp. *leporinum* (35), and the perennial species *H. bulbosum* (17) and *H. secalinum* (7). Ten of the tested 64 arbitrary 10-mer primers amplified polymorphic DNA in all taxonomic units. Analyses was performed within and between populations, species and subspecies. The primers gave a total of 250 RAPD products. The level of polymorphism varied between taxonomic units depending on the primers employed and the plant reproductive system. In general, the most variable were the allogamous species *H. secalinum* and *H. bulbosum* and the autogamous *H. marinum* subsp. *marinum*. Among the amplified bands, 69 (27%) were shared by at least two different taxonomic units. The remaining bands were specific. The results demonstrate differences in the degree of similarity between taxonomic units. Jaccard's similarity coefficients for interval measure within and between populations were used to produce a cluster diagram using the unweighted pair-group method (UPGMA). The different populations of the species and subspecies of *Hordeum* fell into three groups. The first group contained the populations belonging to both subspecies of *H. marinum*, plus those of *H. secalinum*. The populations of *H. marinum* subsp. *gussoneanum* were very closely associated. Those of *H. marinum* subsp. *marinum* were grouped in a broad cluster. The second group, occupying the innermost position of the tree, was very closely associated with the populations of both subspecies of *H. murinum*. The third branch segregated *H. bulbosum*. A series of RAPD markers were investigated by cleaving the amplified products of the same size with restriction endonucleases that recognize targets of 4- or 6-bp. The production of equivalent fragments following cleavage by the same enzyme would seem to demonstrate their homology in samples from different individuals, populations or taxonomic units.

Key words *Hordeum* · Barley · RAPD · Variability · Phylogeny · DNA analyses

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Introduction

Much of the genetic diversity that local, traditional varieties of cultivated crops possessed is being lost. The new varieties are forever more genetically homogeneous and are therefore more exposed to pathogens and adverse environmental conditions (Asins and Carbonell 1989). This has promoted the search for new sources of variation that might be of use in plant-breeding programs, and many national and international organisations have stressed the need for the collection, conservation, and use of wild-species relatives of cultivated species (Brown et al. 1990). Wild populations found within the same agroclimatic environment as cultivated varieties offer the advantage that they are already adapted to living under the same conditions. Their possible use in crossing experiments is therefore of great interest. However, before such work can begin, the genetic variability of these wild populations must be determined (Phillips et al. 1993; Soler et al. 1993).

The realization that DNA sequence polymorphisms between individuals could be used for the analysis of variability has been of major importance. Restriction fragment length polymorphisms (RFLP) are extremely useful for genetic mapping and studies of genetic diversity. Multiple Southern blots, corresponding to hundreds of individuals, can be probed successively and simultaneously (review Tanskley et al. 1989). However, the use of RFLPs in many individuals has certain limitations: the amount of DNA required is relatively large (5–10 μg), and the technique is very expensive and time-consuming. The need for radioactive probes has been overcome with the availability of sensitive non-radioactive detection systems.

Recently, technology for the amplification of discrete sequences, using the polymerase chain reaction (PCR), has offered promising new marker systems for use in the diagnosis of genetic diversity in programs that use many individuals (Saiki et al. 1988). The technique of random amplification of polymorphic DNA (RAPD) is performed using arbitrary sequence oligonucleotide primers on a genomic DNA template (Welsh and McCleland 1990; Williams et al. 1990). The main advantages of RAPD technology are its simplicity of use, reduced running time and lower cost. Moreover, it does not make use of radioactive probes and requires only small amounts of DNA (15–25 ng). However, RAPDs have the problem of limited repeatability, with the confounding factor that repetitive DNA sequences are often amplified (Devos and Gale 1992; Penner et al. 1993). These difficulties may be overcome if care is taken to ensure consistent reaction conditions during amplification. Further, since RAPD markers are dominant, attempts to diagnose genetic diversity have to be designed to take into account the fact that profiles are scored for the presence or absence of a single allele (Williams et al. 1990). The homology of products of the same size resulting from the amplification of DNA of different individuals has also been questioned (Thormann et al. 1994).

The general aim of the present study was to analyse the genetic variability of wild Spanish populations of the genus *Hordeum*. The particular objectives of the experiments decribed in this paper are: (1) to apply RAPD technology to investigate the genetic diversity within and between the natural populations, and to determine the relationships between subspecies and species of the genus; (2) to test the homology of RAPD markers of the same size resulting from amplification by the same DNA primer from different individuals, populations, subspecies and species.

Materials and methods

Plant material

The plant material used in this investigation consisted of 102 populations belonging to six taxonomic units of the genus *Hordeum* growing in Spain. This material is part of the living collection of wild relatives of cultivated *Triticeae* obtained from natural environments over recent years and maintained at the Plant Breeding Unit of the I.N.I.A. (La Canaleja, Madrid, Spain) (Ruiz-Fernández and Soler 1997). In order to conserve the original structure of the natural population, specimens were collected at random and each sample was obtained following the method of Hawkes (1980). About 100 ears were picked from each population in situ. The plant material includes samples from natural populations of two annual species, each represented by two subspecies, and two perennial species, reflecting the presence and distribution of the different taxonomic units in Spain (Fig. 1). The perennials included 17 populations of *H. bulbosum* (2n = 14), collected from the southwest of continental Spain and growing on acidic and neutral soils, and seven populations of *H. secalinum* (2n = 4*x* = 28) growing on humid soil in central Spain. The seven populations of *H. murinum* subsp. *murinum* (2n = 4*x* = 28) were found in habitats at altitudes above 1000 m with high rainfall. The 35 populations of *H. murinum* subsp. *leporinum* (2n = 4*x* = 28) were collected from a range of habitats in the Iberian Peninsula, and in the Balearic Archipielago and Canary Islands. Finally 22 populations of *H. marinum* subsp. *marinum* (2n = 14) and 14 of subsp. *gussoneanum* (2n = 14) were found in different climates in continental Spain and on the Balearic Islands.

DNA extraction and RAPD procedures

The analysis of RAPD was performed using DNA from 15 to 20 plants per population. The DNA was extracted from leaves of 4–6-week-old plantlets as described by Sharp et al. (1988). Each amplification was performed in a reaction volume of 25 μl containing 7.5 ng primer, 12.5 ng total DNA, 2.5 μl reaction buffer, 100 mM dNTPs and 0.5 units of *Taq* DNA Polymerase (Boehringer, Mannheim). The primers used were of 10 bp in length (Operon Technologies – kits E, R, S and T), previously screened in a population diversity project. Amplifications were carried out in a Perkin Elmer 480 thermocycler. The PCR followed the method of Williams

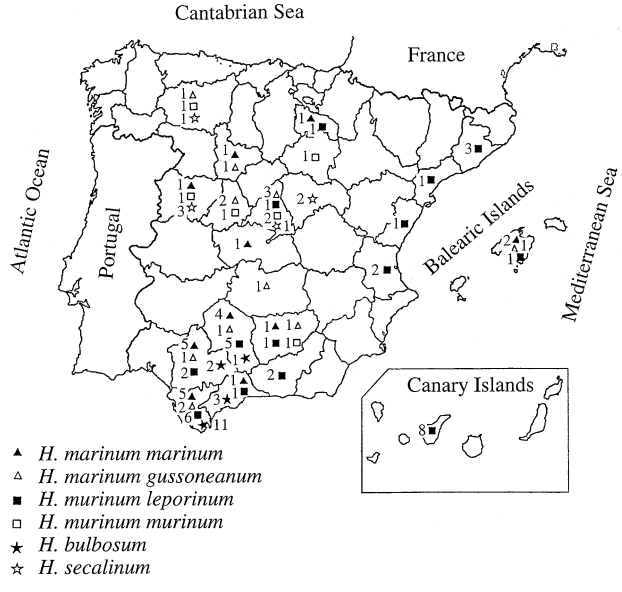

Fig. 1 Origin and classification of material collected for a RAPD survey of natural populations of wild barley growing in Spain and conserved at the Plant Breeding Unit, INIA (La Canaleja, Alcalá, Madrid, Spain)

et al. (1990) with minor modifications: 40 cycles of 1 min at 94°C, 3 min at 36°C and 2 min at 72°C, followed by a final incubation cycle of 5 min at 72°C. Amplification products were then analyzed by electrophoresis in 1.8% agarose gels run at 4.5 V/cm for 2–3 h in a TAE buffer and stained with ethidium bromide. Each reaction and corresponding electrophoresis was repeated at least twice. DNA fragment size-testers from 8 kb to 0.37 kb (Boehringer Mannheim) were run in lateral lanes. The size of each amplification product was automatically estimated using the Bio Image Mod. 60*s*-DNA (Millipore) image analyzer system. Images were captured using a high-resolution scan. Digitalized images were analyzed using BioImage software for RAPD.

DNA digestion

The amplification products were precipitated with ethanol and dissolved in TE as described by Sambrook et al. (1989). DNA samples were then digested with *Taq*I, *Hae*III, *Sau*3AI, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI and *Dra*I (Boehringer, Mannheim). Amplified and restricted DNAs were eletrophoresed in 1.8% agarose gels and 6% acrylamide gels in a TAE buffer, followed by staining with ethidium bromide.

Statistical analysis

The analysis of gels was aided by standardizing experimental conditions and through the use of an Image Analyzer (BioImage of Millipore). Bands of identical size amplified with the same primer were considered to be the same DNA marker. RAPD data were scored in a global matrix that included results of all primers in all materials. The presence (1) or absence (0) of the bands was used for analysis. Statistical analyses of the data were conducted using the rectangular matrix, in which each band (rows) was scored for each taxonomic unit (columns). Analyses were performed by means of the NTSYS computer package (version 1.50, Rohlf 1989). The data matrix was used to compute Jaccard's similarity coefficients following the method of Link et al. (1995) for RAPDs. These were used as operational taxonomic units (OTUs) to construct dendrograms using the unweighted pair group method (UPGMA) (Sneath and Sokal 1973) and the SHAN (sequential, hierarchical, agglomerative and nested clustering) routine (NTSYS software).

The coefficient of variation of each taxonomical unit was estimated using the mean index of similarity and variance between populations.

Results

Ten primers (10-mer) were selected, after assaying 64, to amplify total DNA from samples of all species and/or subspecies. The selection of each primer was based, after several trials, on its suitable amplification of DNA from all species and subspecies. However, results revealing polymorphism in plant samples were ignored during the primer-selection process, in order to avoid a biased estimation of variability (Clark and Lanigan 1993). Special care was taken to eliminate variations in DNA concentration, and to ensure consistent reaction conditions and thermal profiles during amplification. All bands consistent in duplicate PCR experiments were scored as representative markers of the genomic DNA of the corresponding taxonomic group.

Interpopulational and interspecific analyses

The selected primers amplified a total of 250 DNA products that were very useful in distinguishing species and/or subspecies (Table 1). The total number of amplified bands per primer varied from 8 (EO3) to 49 (R10). The size of the products varied from 376 bp (S13) to 3200 bp (T18). Primers R10 and T16 gave more products and more polymorphic markers than all others. Different populations from the same species or subspecies showed rather constant amplified products and RAPD patterns for the same primer (Fig. 2). Very few RAPD bands were differentially present in different populations of the same taxonomic group. The level of

Table 1 Primers used and number of RAPD markers obtained in the six taxonomic units of the genus *Hordeum* investigated

Primers	Sequence 5′ → 3′	RAPD markers (total no.)	Size (bp) min–max		No. polymorphic bands (PB) and percentage of polymorphic markers (%P)											
					H. bulbosum		*H. marinum* ssp. *marinum*		*H. marinum* ssp. *gussoneanum*		*H. murinum* ssp. *murinum*		*H. murinum* ssp. *leporinum*		*H. secalinum*	
					PB	%P	PB	%P	PB	%P	PB	%P	PB	%P	PB	%P
E-01	CCCAAGGTCC	24	725	2050	3	42	5	71	0	0	0	0	2	66	2	22
E-03	CCAGATGCAC	8	445	1165	2	50	0	0	0	0	0	0	0	0	0	0
R-01	TGCGGGTCCT	23	646	3196	2	33	3	37	0	0	1	25	0	0	2	50
R-04	CCCGTAGCAC	18	637	2130	0	0	4	80	0	0	2	40	2	40	0	0
R-10	CCATTCCCCA	49	425	2832	10	83	14	93	3	42	2	25	13	92	9	81
S-13	GTCGTTCCTG	21	376	2690	2	66	1	25	0	0	4	50	7	77	1	20
T-01	GGGCCACTCA	12	623	2638	1	33	0	0	0	0	2	50	2	50	0	0
T-07	GGCAGGCTGT	21	589	1662	2	50	5	83	2	40	2	50	4	80	2	66
T-16	GGTGAACGCT	41	770	2742	11	100	6	85	3	60	3	50	4	57	11	100
T-18	GATGCCAGAC	33	560	3200	4	50	16	100	0	0	5	71	5	71	3	60
Totals		250			37	60	54	76	8	21	21	42	39	66	30	55

polymorphism shown by each primer was variable for the different taxonomic units. The populations of *H. marinum* subsp. *marinum* showed the greatest variation (76% polymorphic bands). The lowest interpopulational variation corresponded to *H. marinum* subsp. *gussoneanum* (21% polymorphic bands).

Polymorphism was rather high between different species. Only four amplified bands were shared by all the species and subspecies analyzed, and only one of them was consistently present in all the 102 populations studied. The distribution of RAPD bands in each taxonomic unit is listed in Table 2. Of the amplified products, 69 (27%) were shared by at least two different taxonomic units. The remaining bands were specific. Different results obtained for related subspecies are worthy of note. Thus, the subspecies *marinum* and *gussoneanum* of the species *H. marinum* shared only seven RAPD markers, a figure lower than the number of amplified products held in common *by H. marinum* subsp. *gussoneanum* and *H. secalinum* (16). On the other hand, the subspecies *murinum* and *leporinum* of the species *H. murinum* shared many more bands (49). These results seem to demonstrate differences in the degree of similarity between the subspecies of both species.

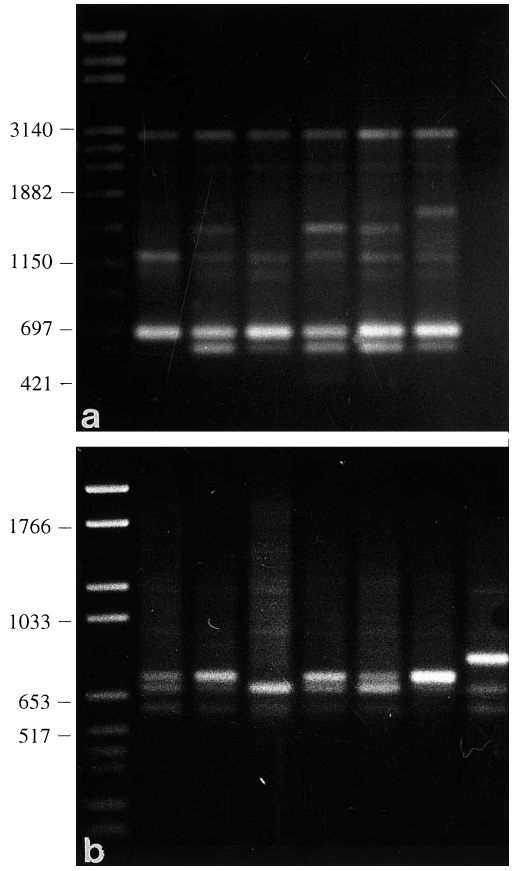

Fig. 2 RAPD profile showing interpopulational polymorphism. **a** Populations of *H. bulbosum* amplified using the primer T16. The *left lane* includes the molecular-weight-marker IV (Boehringer Mannheim). **b** Populations of *H. murinum* subsp. *leporinum* amplified using the primer T01. The *left lane* includes the molecular-weight-marker VI (Boehringer Mannheim)

These conclusions were confirmed by statistical analysis. A data matrix was generated from the RAPD results. This was used to compute an output matrix of Jaccard's similarity coefficients for interval-measure within populations of all the taxonomic units. The coefficients were used to produce a cluster using the unweighted pair-group method (UPGMA). This takes into consideration all the arithmetic averages of species and/or subspecies. The data derived from the analysis of similarity were used to construct a dendrogram (Fig. 3). The species and subspecies of *Hordeum* included in this study fell into three groups. The first group associated the subspecies of *H. marinum* with *H. secalinum*. All the populations of *H. marinum* subsp. *gussoneanum* were gathered into a very narrow cluster. By contrast, the populations of *H. marinum* subsp. *marinum* formed a broad cluster. The remainder of the tree can be regarded as two broad clusters. The long cluster, occupying the innermost position of the tree, associates very closely with the populations of both subspecies of *H. murinum*. The third branch segregates *H. bulbosum*.

The coefficients of variation between populations and within subspecies and species were estimated (Table 3). Surprisingly, the most widely separated coefficient values coincided in different subspecies of the same species. Thus, within *H. marinum*, the subsp.

Table 2 Number of RAPD products generated (diagonal) and shared (remaining data) by ten primers in 102 populations of six taxonomic units of *Hordeum*. Between brackets are registered the number of specific markers found in each taxonomic unit

Taxonomic unit	*H. bulbosum*	*H. marinum* ssp. *marinum*	*H. marinum* ssp. *gussoneanum*	*H. murinum* ssp. *murinum*	*H. murinum* ssp. *leporinum*	*H. secalinum*
H. bulbosum	61 (54)	5	5	6	6	5
H. mar. ssp. *marinum*		71 (61)	7	5	5	8
H. mar. ssp. *gussoneanum*			38 (21)	5	5	16
H. mur. ssp. *murinum*				50 (1)	49	6
H. mur. ssp. *leporinum*					59 (10)	6
H. secalinum						54 (34)

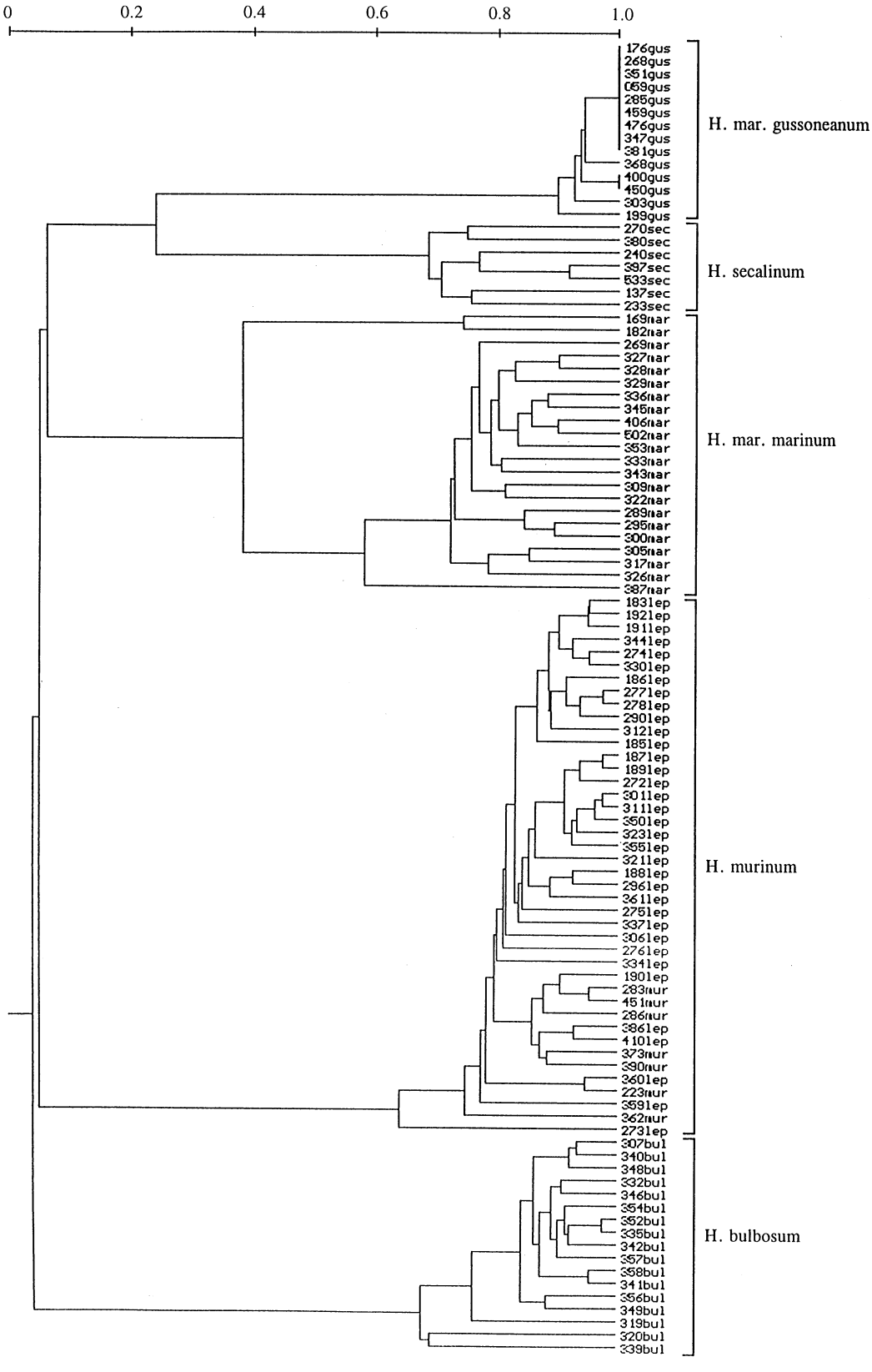

Fig. 3 Dendrogram illustrating genetic relationships among 102 populations of six species or subspecies of the genus *Hordeum*. Generated by the UPGMA cluster analysis (NTSYS) calculated from 250 RAPD markers produced by ten single primers

Table 3 Analysis of molecular diversity. Coefficients of variation and variance for each taxonomic unit based on the similarity indices between populations

Taxonomic unit	Mean	Variance	Coef Var
H. bulbosum[a]	0.836882	0.010512	12.25113
H. marinum ssp. *marinum*[b]	0.706206	0.029751	24.42397
H. marinum ssp. *gussoneanum*[b]	0.961737	0.001713	4.30289
H. murinum ssp. *murinum*[b]	0.852921	0.011476	12.55991
H. murinum ssp. *leporinum*[b]	0.830986	0.006202	9.47713
H. secalinum[c]	0.788024	0.017648	16.85805

[a] Strictly allogamous
[b] Mainly autogamous
[c] Mainly allogamous

Table 4 The analysis of intrapopulational variation in each taxonomic unit on that deduced from the application of six primers to study 20 individuals per population

Primers	RAPD products (TB) , polymorphic markers (PB) and percentage of variable bands (%P)																	
	H. bulbosum Pop. 339			*H. marinum* ssp. *marinum* Pop. 309			*H. marinum* ssp. *gussoneanum* Pop. 351			*H. murinum* ssp. *murinum* Pop. 362			*H. murinum* ssp. *leporinum* Pop. 275			*H. secalinum* Pop. 240		
	TB	PB	%P	TB	PB	%P	TB	PB	%P	TB	PB	%P	TB	PB	%P	TB	PB	%P
R-01	4	0	0	7	2	28	2	0	0	4	1	25	4	0	0	3	0	0
R-04	3	2	66	2	1	50										2	0	0
R-10										8	3	37	6	1	16			
S-13				4	2	50	4	0	0							5	1	20
T-07	3	1	33	3	1	33	4	0	0	3	2	66	3	1	33			
T-16	5	0	0				3	0	0	4	2	50	3	1	33	5	1	20
Total	15	3	20	16	6	37	13	0	0	19	8	42	16	3	18	15	2	13

gussoneanum and the subsp. *marinum* presented the lowest and highest levels of interpopulational variation respectively. The remainder of the taxonomic units showed intermediate values.

Intrapopulational homogeneity was also surveyed by testing individuals of one population of each taxonomic unit. Twenty individual plants of each population were scored with four different primers (Table 4). In intrapopulational analyses, the level of polymorphism detected between the individuals of each population depended on the taxonomic unit studied (Fig. 4). The level of intrapopulational diversity is also revealed by the coefficient of variation registered in Table 5. For instance, all the individuals of population 351 (*H. marinum* subsp. *gussoneanum*) showed the same RAPD profiles for all the primers assayed. On the other hand, the individuals of populations 309 (*H. marinum* subsp. *marinum*) and 362 (*H. murinum* subsp. *murinum*) showed a significantly higher level of variability. The populations of the allogamous species *H. bulbosum* and *H. secalinum* (339 and 240), showed a relatively high level of polymorphism. This was particularly high in *H. bulbosum*. This species is strictly allogamous due to the existence of a genetic-incompatibility system (Bothmer et al. 1991). The high coefficient of variation obtained in *H. marinum* subsp. *marinum* disagrees with the low intrapopulational genetic variability that would be expected according to its autogamous reproductive system (Hamrick 1990; Wolff 1991).

Homology of discrete products of the same size, amplified from DNA of different individuals, populations or taxonomic units

The lack of information on the sequence of amplified products and the problems associated with the reproducibility of RAPD technology have led to certain doubts with respect to the homology of RAPD markers of the same size. Homology can be probed by cloning and sequencing the amplified products, or using them as DNA probes in Southern experiments. However, these procedures are difficult and time-consuming, and therefore unsuitable for the management of populations.

A helpful alternative for the testing of homology is the use of restriction endonucleases to cleave amplified products of the same size. This is based on the principle of the existence of common targets in twin positions of homologous sequences (Chapco et al. 1992). The production of equivalent fragments substituting for the original amplified products of DNA on agarose gels is

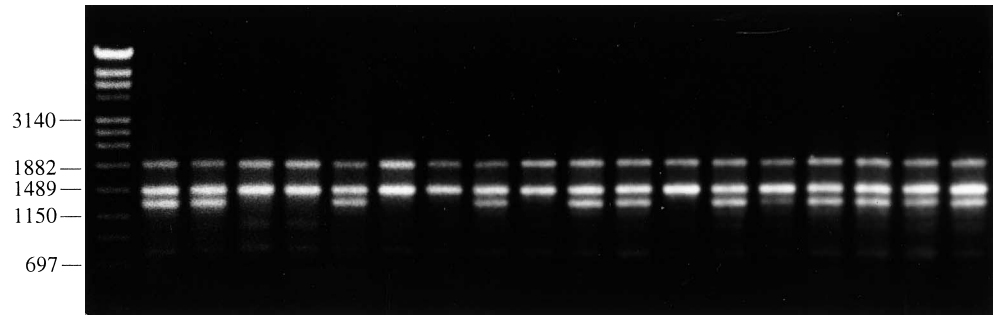

Fig. 4 RAPD profile showing intrapopulational polymorphism. The *left lane* includes the molecular-weight-marker IV (Boehringer Mannheim). The second line shows the RAPD profile from bulk DNA. Individuals belonging to population 275 (*H. murinum* subsp. *leporinum*) using the primer T16 (lanes 3 to 19)

Table 5 Coefficients of variation and variance for each taxonomic unit based on the mean of the similarity indexes between the individuals of each population

Population	Mean	Variance	Coef Var
339 (*H. bulbosum*)	0.914954	0.003686	6.63534
309 (*H. mar.* ssp. *marinum*)	0.881579	0.030384	19.77260
351 (*H. mar.* ssp. *gussoneanum*)	1	0	0
362 (*H. mur.* ssp. *murinum*)	0.853561	0.017752	15.60936
275 (*H. mur.* ssp. *leporinum*)	0.934091	0.002899	5.75496
240 (*H. secalinum*)	0.957576	0.003183	5.89208

consistent with the assumption of homology. The accuracy of this conclusion is reinforced when more than one enzyme analysis is extended to a significant number of RAPD markers shared by different individuals, populations, species and subspecies.

In the present study, different enzymes that recognize targets of 4-bp or 6-bp were used with the amplified DNA of individuals from the same and different taxonomic units and populations. Figures 5 to 7 show examples of DNA of different origin digested with a number of restriction enzymes. In all cases, amplified bands of the same size belonging to individuals of the same or different populations, and which were also digested by the same enzyme, gave identical results. The RAPD products were either uncleaved or else cleaved to give equivalent fragments. These results seem to demonstrate the homology of amplified DNA from different individuals. They also increase the level of confidence one might have in RAPD markers. Finally, the use of restriction endonucleases offers an increase in the number of molecular markers available for the study of genetic diversity with respect to the direct system of RAPD. The application of this methodology was more complex in the case of amplified DNA belonging to different taxonomic units (Fig. 7). However, only 2 out of 21 markers tested from the RAPD products shared by two or more different subspecies or species gave fragments of different size after digestion with the same restriction enzyme.

Discussion

Yang and Quiros (1993) used RAPD technology to characterize celery cultivars. They estimated that bulk DNA from 5 to 20 plants gave a compound estimation of dispersed markers depending on the genetic homogeneity of each species. A comparison of the RAPD markers from the populations of the present study was performed on the amplification of bulk DNA from 10 to 20 plants. The validity of the sample size was verified comparing the RAPD profiles of the bulk DNA with the profiles of 15 to 20 individuals of the same population. The phenotypic variation of the amplified products in the DNA from different individuals was represented in the RAPD profile of the bulk DNA (see Fig. 4). Only a few faint bands observed in individual phenotypes were absent in the RAPD profile from the bulk DNA. This can be explained by differences in the concentration of individual and bulk DNA in the regions homologous to each primer. Rafalski et al. (1991) postulated that, in RAPD reactions, the composition of the amplification products is determined by a competition between potential priming sites in the template rather than by the total number of priming sites available. DNA from individuals and bulk DNA may offer differences in the relative level of stringency of annealing conditions for different priming sites, giving distinct degrees of matching and varying intensity in RAPD bands. Supporting evidence for this assertion has been obtained by other authors (Wilkie et al. 1993; Brummer et al. 1995; Loarce et al. 1996).

The balance of shared bands and specific bands within and between the natural populations of the *Hordeum* material here studied, indicates that useful phylogenetic information could be gained from the analysis of common bands. However, before using this information, it is important to confirm whether bands which co-migrate in different RAPD profiles represent homologous sequences, or whether they are simply fortuitous bands of similar size (Thormann et al. 1994).

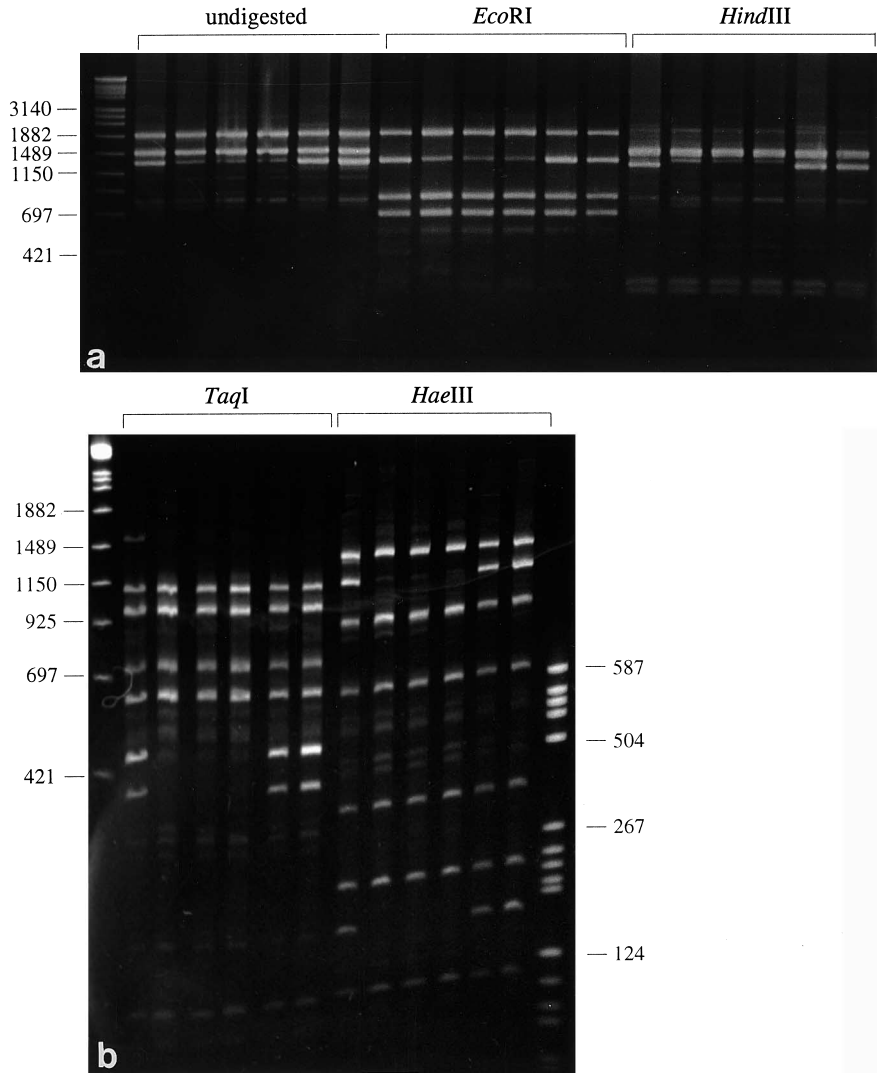

Fig. 5 **a, b** Products of the amplification of six individuals from population 275 (*H. murinum* subsp. *leporinum*) using the primer T16 and digested with different restriction endonucleases. **a** Agarose gel (1.8%) showing the RAPD profile obtained without digestion (*lanes 2 to 7*) and after digestion with *Eco*RI (*lanes 8 to 13*) and *Hin*dIII (*lanes 14 to 19*). The *left lane* includes the molecular-weight-marker IV (Boehringer Mannheim). **b** Acrylamide gel (6%) showing the fragments produced by the endonucleases *Taq*I (*lanes 2 to 7*) and *Hae*III (*lanes 8 to 13*). The *left and right lanes* include the molecular-weight-markers IV and V (Boehringer Mannheim), respectively

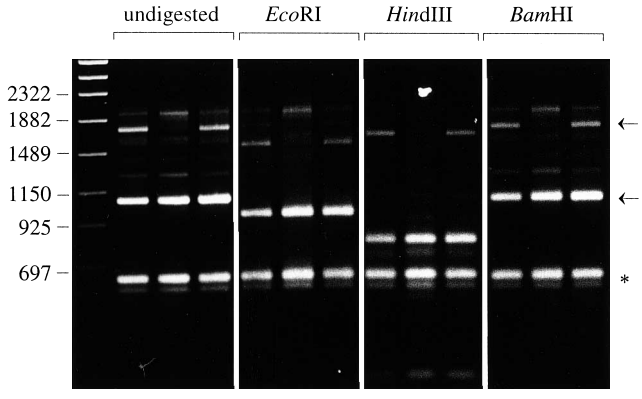

Fig. 6 Agarose gel (1.8%) showing RAPD profile of bulk DNA from the populations 59, 176 and 347 of *H. marinum* subsp. *gussoneanum* using primer R04, without digestion (*lanes 2 to 4* from left to right) and after digestion with different restriction endonucleases. The *left lane* includes the molecular-weight-marker IV (Boehringer Mannheim). The *arrows* mark digested products that supplied fragments of identical size. * = undigested bands

This has been studied previously by investigating a small sample of different bands using Southern analysis of RAPD gels, and employing single isolated bands as probes (Chalmers et al. 1992; Wilkie et al. 1993; Thormann et al. 1994). This is a difficult technique which is only really useful for analyzing particular bands that are rare or which are critical polymorphic markers (Tinker et al. 1993). Southern analysis then became unavailable for investigating large samples of bands. In the present study an alternative method was used that consisted of treating amplified DNA from comparative samples with the same restriction enzymes. This method permitted the analysis of up to 70 different amplified products, representing 30% of the total amplified products obtained with ten primers. The results demonstrate that only 10% of the amplified products that co-migrated in different taxonomic units differ in the presence of a target for the same restriction enzyme. This result does not absolutely exclude the homology of co-migrated RAPD markers, but demonstrates the

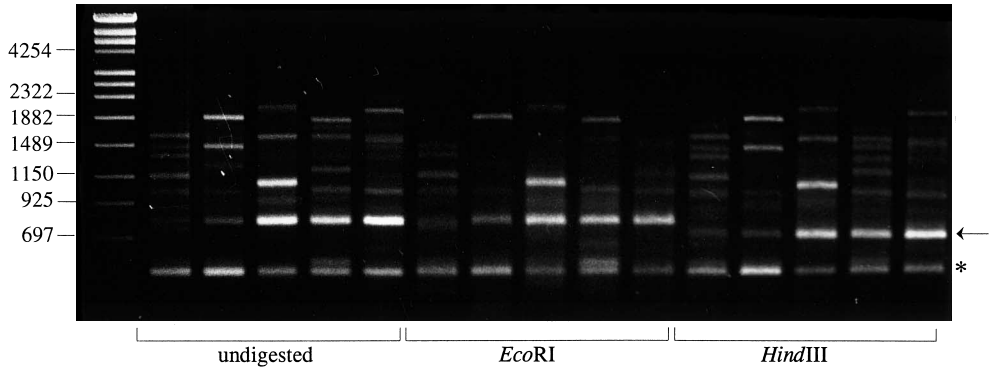

Fig. 7 Agarose gel (1.8%) showing RAPD profiles using primer T07 of bulk DNA from the populations 189 of *H. murinum* subsp. *leporinum* (*lanes 2, 7 and 12*), 346 of *H. bulbosum* (*lanes 3, 8 and 13*), 333 of *H. marinum* subsp. *marinum* (*4, 9 and 14*), 380 of *H. secalinum* (*5, 10 and 15*), and 368 of *H. marinum* subsp. *gussoneanum* (*lanes 6, 11 and 16*), without digestion (*lanes 2 to 6*) and after digestion with different restriction endonucleases (*lanes 7 to 15*). The *left lane* includes the molecular-weight-marker IV (Boehringer Mannheim). The *arrow* marks fragments of identical size obtained by digestion with *Hin*dIII of the RAPD marker of 754 bp. * = undigested bands

non-existence of a common target in the sequence. Even so, this figure is not very different to the 20% of non-homologous co-migrating bands reported by Thormann et al. (1994) in a Southern-blot study of genetic relationships within and between species of the genus *Brassica*. Using the same technique, these authors demonstrated that the amplified products that co-migrated in different individuals from the same species were homologous in all cases.

The results of the present study, using restriction enzymes to digest amplified DNA from different individuals of the same species, agrees well with the Southern results obtained in other material. All the common bands showed the same behaviour with respect to the enzymes. They were scored either as uncleaved by all enzymes or as cleaved by the same enzyme. Moreover, enzymes were used that recognized targets of 4 bp, which consequently have a major probability of cleaving the genome. The findings of similar results with eight different enzymes is consistent with the hypothesis of the homology of co-migrated bands.

The level of intra- and inter-populational variation detected with the RAPD markers was different for each taxonomic unit, partially depending on the reproductive system of each of them. The extreme values of inter-populational variation corresponded to the subspecies of the autogamous species *H. marinum*. These subspecies also shared a low proportion of common RAPD bands. At the interpopulational level, the allogamous species *H. secalinum* and *H. bulbosum* showed intermediate values of variation with respect to both subspecies of *H. marinum*, but higher than that of *H. murinum*. A good correlation between the level of intraspecific variation and the reproductive system has been generally observed for RAPDs markers in other species (Kazan et al. 1993; Jain et al. 1994; Wachira et al. 1995). In the present study, the only exception to this general rule was *H. marinum* subsp. *gussoneanum*, which shows an exceptionally low interpopulational coefficient of variation. The results in this taxonomic unit suggest that, despite the wide geographical distribution of the 14 populations studied, the incidence of RAPD-type polymorphism is lower than in the other species and subspecies. A similar situation was observed by Chalmers et al. (1992) for RAPD markers in allogamous species of the genus *Gliricidia*.

The present study demonstrates that analysis of RAPD markers can be used successfully to study intraspecific diversity and phylogenetic relationships between different species of *Hordeum*. The results demonstrate a rather high coincidence in RAPD markers between *murinum* and *leporinum*. Consequently both subspecies of *H. murinum* appear closely related. These results, and the correspondence with the physical maps obtained by FISH (De Bustos et al. 1996), strongly suggest, as proposed by Linde-Laursen et al. (1989), that subsp. *murinum* and *leporinum* are so closely related that they might even be considered as a single taxon. In contrast, the divergence observed between *marinum* and *gussoneanum* suggests a clear separation that questions their taxonomic placement in a monotypic group with specific rank. The use of FISH on the chromosomes of both subspecies of *H. marinum* has also demonstrated substantial differences in the pattern of distribution of hybridization sites (De Bustos et al. 1996). These results agree with previous investigations that consider both taxonomic units as at least subspecies of *H. marinum* (Newski 1941; Bothmer and Jacobsen 1985; Symeonidis and Moustakas 1986; Bothmer et al. 1989), or even as separate species (Jorgensen 1986).

The observation that different genetic systems reveal different degrees of polymorphism can be explained by the very nature of the genetic markers. Thus, isozymes and proteins are products of structural genes,

submitted to more or less strict selective processes which increase their efficiency. However, RAPD amplification techniques scan anonymous DNA-sequences, whether they are, or are not, coding regions, whether unique or repetitive, and which are randomly distributed in the genome. As a consequence, RAPD technology is less restrictive in respect of the polymorphism of complete genomes and provides researchers with an efficient screening method for DNA sequence-based polymorphism at a very large number of loci.

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References

Asins MJ, Carbonell EA (1989) Distribution of genetic variability in a durum wheat world collection. Theor Appl Genet 77 : 287–294

Bothmer R, Jacobsen N (1985) Origin, taxonomy and related species. In: Rasmusson D (ed) Barley. Agronomy Monograph No. 26, ASA, pp 19–26

Bothmer R, Flink J, Jacobsen N, Jorgensen RB (1989) Variation and differentiation in *Hordeum marinum* (Poaceae). Nord J Bot 9 : 1–10

Bothmer R von, Jacobsen N, Baden C, Jrgensen RB, Linde-Laursen I (1991) An ecogeographical study of the genus Hordeum. Systematic and ecogeographic studies on crop gene pools 7, IBPGR, Rome

Brown ADH, Burdon JJ, Grace JP (1990) Genetic structure of *Glycine canescens*, a perennial relative of soybean. Theor Appl Genet 79 : 729–736

Brummer EC, Bouton JH, Kochert G (1995) Analysis of annual *Medicago* species using RAPD markers. Genome 38 : 362–367

Chalmers KJ, Waugh R, Sprent JI, Simons AJ, Powell W (1992) Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. Heredity 69 : 465–472

Chapco W, Ashton NW, Martel RKB, Antonishyn N, Crosby W (1992) A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. Genome 35 : 569–574

Clark AG, Lanigan CMS (1993) Prospects for estimating nucleotide divergence with RAPSs. Mol Biol Evol 10 : 1096–1111

De Bustos A, Cuadrado A, Soler C, Jouve N (1996) Physical mapping of repetitive DNA sequences and 5*s* and 18*s*-26*s* rDNA in five wild species of the genus *Hordeum*. Chromosome Res 4 : 491–499

Devos KM, Gale MD (1992) The use of random amplified polymorphic DNA markers in wheat. Theor Appl Genet 84 : 567–572

Hamrick JL (1990) Isozymes and the analysis of genetic structure in plant populations. In: Soltis ED, Soltis PS (eds) Isozymes in plant biology. Chapman and Hall, London, pp 87–105

Hawkes JG (1980) Crop genetic resources. Field collection manual for seed crops, root and tuber crops, tree fruit crops and related wild species. International Board for Plant Genetic Resources (I.B.P.G.R.) and the European Association for Research on Plant Breeding (Eucarpia), Rome

Jain A, Bhatia S, Banga SS, Prakash S (1994) Potential use of the random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationships to heterosis. Theor Appl Genet 88 :116–122

Jörgensen RB (1986) Relationships in the barley genus (*Hordeum*): an electrophoretic examination of proteins. Hereditas 104 : 273–291

Kazan K, Manners JM, Cameron DF (1993) Genetic variation in agronomically important species of Stylosanthes determined using random amplified polymorphic DNA markers. Theor Appl Genet 85 : 882–888

Linde-Laursen I, Bothmer R von, Jacobsen N (1989) Giemsa C-banded karyotypes of *Hordeum marinum* and *H. murinum*. Genome 32 : 629–639

Link W, Dixkens C, Singh M, Schwall A, Melchinger (1995) Genetic diversity in European and Mediterranean faba bean germ plasm revealed by RAPD markers. Theor Appl Genet 90 : 27–32

Loarce Y, Gallego R, Ferrer E (1996) A comparative analysis of the genetic relationships between rye cultivars using RFLP and RAPD markers. Euphtyica 88 : 107–115

Newski SA (1941) Beitrage zur Kenntniss der wildwachsenden Gersten in Zusammenhang mit der Frage über der Ursprung von *Hordeum vulgare* L. Und *Hordeum distichon* L. (Versuch einer Monographie der Gattung *Hordeum*). Today Bot Int Akad Nauk SSSR. Ser 1,5 : 20–21

Penner GA, Chong J, Levesque-Lemay M, Molnar SI, Fedak KG (1993) Identification of a RAPD marker linked to the oat stem rust gene *Pg3*. Theor Appl Genet 85 : 702–705

Phillips TD, Murphy JP, Godman MM (1993) Isozyme variation in germplasm accesions of the wild oat *Avena sterilis* L. Theor Appl Genet 86 : 54–64

Rafalski JA, Tingey SV, Williams JGK (1991) RAPD markers – a new technology for genetic mapping and plant breeding. Agric Biotech News Inf 3 : 645–648

Rohlf JF (1989) NTSYS. pc: numerical taxonomy and multivariate analysis system for the IBM pc microcomputer (and compatibles). In: Setavek. Applied Biostatistics Inc, N.Y.

Ruiz-Fernández J, Soler C (1997) Distribution and habitat of Spanish populations of the subtribe Hordeineae; improved views following germplasm collecting activities. Genet Res Crop Evol 44 : 33–41

Saiki RK, Gelfond DH, Stoffel S, Scharf SJ, Higuchi R, Horn BT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 : 487–491

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sharp PJ, Desai S, Gale MD (1988) Isozyme variation and RFLPs at the β-amylase loci in wheat. Theor Appl Genet 76 : 691–699

Sneath PHA, Sokal RR (1973) Numerical taxonomy. Freeman, San Francisco

Soler C, Galindo,C, González-Castaño S, Casanova C, García P (1993) Isozyme variation in Spanish natural populations of *Elytrigia pungens* (Pers.) Tutin and *Elytrigia repens* (L). Nevski. Heredity 71 : 51–58

Symeonidis L, Moustakas M (1986) Biosystematic study of the *Hordeum murinum* group in Greece. Flora 178 : 177–182

Tanksley SD, Young ND, Patterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7 : 57–264

Thormann CE, Ferreira ME, Camargo LEA (1994) Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theor Appl Genet 88 : 973–980

Tinker NA, Fortin MG, Miller DE (1993) Random amplified polymorphic DNA and pedigree relationships in spring barley. Theor Appl Genet 85 : 976–984

Wachira FN, Waugh R, Hackett CA, Powell W (1995) Detection of genetic diversity in tea (*Camelia sinensis*) using RAPD markers. Genome 38 : 201–210

Welsh J, McClelland J (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18 : 7213–7218

Wilkie SE, Isaac PG, Slater RJ (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 86 : 497–504

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18 : 6531–6535

Wolff K (1991) Analisys of allozyme variability in the three *Plantago* species and a comparison to morphological variability. Theor Appl Genet 81 : 119–126

Yang X, Quiros C (1993) Identification and classification of celery cultivars with RAPD markers. Theor Appl Genet 86 : 205–212