BIOLOGIA PLANTARUM 39 (4): 543-552, 1997

Identification of wild and cultivated *Hordeum* species using two-primer RAPD fragments

P.V. REDDY* and K.M. SOLIMAN

Department of Plant and Soil Science, Alabama A&M University, P.O. Box 1208, Normal, AL 35762, USA

Abstract

Random amplified polymorphic DNAs (RAPD) analysis has been adapted to assess the degree of RAPD polymorphism within the genus *Hordeum* to determine if this approach can distinguish wild and cultivated species. Nineteen wild and seven cultivated accessions were evaluated using 4 random 10-mer primers. The potential of the RAPD assay was further increased by combining two primers in a single polymerase chain reaction (PCR). RAPD fragments generated by two pairs of arbitrary 10-mer primers discriminated six wild species and one cultivated species by banding profiles. The size of the amplified DNA fragments ranged from 150 to 2300 base pairs. 33 % percent of the fragments were common to both wild and cultivated species; 67 % were specific to either wild or cultivated species. The average difference in fragments was less within the species than among the species. By comparing RAPD fingerprints of wild and cultivated barley, markers were identified among the set of amplified DNA fragments which could be used to distinguish wild and cultivated *Hordeum* species.

Additional key words: barley, DNA polymorphism, polymerase chain reaction.

Introduction

Genetic markers are routinely used for genome analysis in a number of crop species. Traditional cultivar identification based on morphological traits requires extensive observations of mature plants and, in many situations, lacks definition and objectivity (Wrigley *et al.* 1987). Furthermore, morphological traits often can not serve as

Received 21 March 1996, *accepted* 10 May 1996.
Acknowledgments: Contributed by the Agricultural Experiment Station, Alabama Agricultural and Mechanical University, Journal No T 233. The authors wish to thank Drs. D. Hoffman and A. Zipf for their helpful suggestions. This research was supported in part by the National Science Foundation RIMI grant 88-050-97.
*New address: Dept. of Plant Pathology, Foran Hall, Cook College, Rutgers University, New Brunswick, NJ 08903, USA. Fax (+1) 908-932-9311; e-mail veera@rci.rutgers.edu.

unambiguous markers because of environmental influences. To solve this problem of ambiguity, specific molecular markers have been successfully developed in the last two decades.

Restriction fragment length polymorphism (RFLP) markers are powerful tools for genetic analysis (Tanksley *et al.* 1989). The flow of desirable genes to members of a segregating population can be traced with RFLP markers (Slocum *et al.* 1990, Bonierbale *et al.* 1988). RFLPs have been used to generate extensive genetic maps of several important plant species over periods of several months as opposed to the years required using comparable morphological markers. But the primary disadvantages of using RFLPs in plant genetic resource characterization are the cost of materials and labor, the processing time, and the use of radioactive materials.

Polymerase Chain Reaction (PCR) methodology has impacted molecular biology research perhaps more than any other technique in recent years. Williams *et al.* (1990) and Welsh and McClelland (1990) reported a novel technique based on the amplification of random DNA sequences by PCR with short arbitrary primers. Unlike the other PCR based techniques, this new assay does not require any specific sequence information on the target genome. Random amplified polymorphic DNAs (RAPDs) segregate in Mendelian fashion (Williams *et al.* 1990); therefore, they can be used effectively as genetic markers. In RAPD mapping, decamer oligonucleotide primers of arbitrary sequence with a guanine cytosine (GC) content of above 50 % are used to randomly amplify segments of genomic DNA. The advantages of this technique are its ability to detect extensive polymorphisms, simplicity, rapidity, and the capability to work with very small amounts of genomic DNA.

Barley (*Hordeum vulgare* L.) is one of the world's oldest domesticated crops, and probably shares with wheat the distinction of being the first wild plant form brought under cultivation. *H. spontaneum* L. is the most likely wild progenitor of cultivated *H. vulgare*. Hybridization has played a major role in the evolution of *Hordeum* species. Both autopolyploidy, as seen in *H. bulbosum* L., and various kinds of segmental allopolyploidy are known in the genus (Bothmer 1979, Jorgenson 1982, Bothmer *et al.* 1989b). The nature of the polyploidy is in many cases difficult to analyze with conventional cytogenetic methods (Bothmer *et al.* 1989a). For *Hordeum*, there is presently an extensive body of biosystematic data based on cytogenetic, biochemical, and molecular analyses (Bothmer *et al.* 1986, Jorgenson 1986, Gupta *et al.* 1989). *H. bogdani* (Wil.), *H. bulbosum*, *H. californicum* (Stebbins), *H. muticum* (Hunziker) Presl. and *H. procerum* (Hunziker) are some of the wild species. Clegg *et al.* (1984) and Neale *et al.* (1986) observed variation among cultivated and wild barley populations based on their chloroplast DNA restriction fragment length polymorphism. In this study, we adapted the RAPD assay for the detection of polymorphic DNA fragments in *Hordeum*. Our specific objectives were to determine the potential for using this approach to generate polymorphic fragments for species identification and phylogenetic analysis.

Materials and methods

Plants: Accessions of 6 wild and 1 cultivated *Hordeum* species were obtained from Dr. K.B. Jensen, Utah State University, Logan, UT (Table 1), and were scored for the presence or absence of RAPD fragments. The accessions were randomly chosen.

Table 1. Wild and cultivated *Hordeum* species surveyed for RAPDs.

Serial number	Accession number	Species	Chromosome number	Origin
1.	PI 269406	*H. bogdani*	14	Afghanistan
2.	PI 314696	*H. bogdani*	14	Russia
3.	PI 401386	*H. bogdani*	14	Iran
4.	PI 440413	*H. bogdani*	14	Russia
5.	PI 318649	*H. bulbosum*	14	Greece
6.	PI 343189	*H. bulbosum*	28	Russia
7.	PI 440415	*H. bulbosum*	28	Russia
8.	PI 440417	*H. bulbosurn*	28	Russia
9.	PI 440418	*H. bulbosum*	28	Russia
10.	PI 531776	*H. bulbosurn*	28	Hungary
11.	PI 53 1778	*H. californicum*	14	USA
12.	PI 53 1779	*H. californicum*	14	USA
13.	PI 531785	*H. muticum*	14	Argentina
14.	PI 531787	*H. procerum*	42	Argentina
15.	PI 284744	*H. spontaneum*	14	Israel
16.	PI 296897	*H. spontaneum*	14	Israel
17.	PI 284744	*H. spontaneum*	14	Israel
18.	PI 296897	*H. spontaneum*	14	Israel
19.	PI 382247	*H. spontaneum*	14	Israel
20.	DL 71	*H. vulgare*	14	USA
21.	NK 1467	*H. vulgare*	14	USA
22.	NK 1208	*H. vulgare*	14	USA
23.	KM 614	*H. vulgare*	14	USA
24.	KM 615	*H. vulgare*	14	USA
25.	Arlo	*H. vulgare*	14	USA
26.	NK 1272	*H. vulgare*	14	USA

DNA extraction: Plants were grown in a greenhouse. Five plants were sampled from each accession, and total cellular DNA of the pooled sample was prepared as described by Murray and Thompson (1980) and Saghai-Maroof *et al.* (1984). DNA concentration was determined from the absorbance measured at 260 nm with a *Beckman* spectrophotometer (*DU-64*, Fullerton, USA).

DNA amplification: Four arbitrary decamer primers (E01, E02, F01, and F09) purchased from *Operon Technologies* (Alameda, USA) were used for PCR amplification following the protocol reported by Williams *et al.* (1990) with minor modifications. The reaction components were reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.01 % Triton X-100], 200 μM dNTP, 0.2 μM primer,

2.5 units of Taq polymerase, 50 ng of genomic DNA and 1.0 mM $MgCl_2$ in a 0.1 cm^3 reaction mixture. The Taq polymerase, dNTPs and reaction buffer were purchased from *Promega* (Madison, USA). For the DNA amplification, a *Perkin Elmer Cetus* (Norwalk, USA) DNA thermal cycler was programmed for 45 cycles of 94 °C for 1 min denaturing, 36 °C for 1 min annealing and 72 °C for 2 min primer extension. After amplification, 0.03 cm^3 of the samples were loaded on 2.0 % agarose gels in TBE buffer and run at 4 V cm^{-1}. A 100 base pairs (bp) DNA ladder (BRL) was used as a molecular standard. The gels were stained in 0.5 μg cm^{-3} ethidium bromide solution for 20 min, destained with tap water for 10 min and photographed under 260 nm UV radiation with *Polaroid* type *55* film. Each amplified product was identified by its size in base pairs along with the primers used in the reaction. For example, E 1,2-1800 refers to the 1800 bp product amplified with primers E01 and E02. Since non-specific amplification is a common occurrence with PCR under relaxed conditions such as RAPD, the experiment was repeated at least twice. Only the reproducible bands in multiple runs, regardless of their intensity were considered in the study. The profiles of the amplified products from each wild and cultivated species were compared to each other for identification of specific markers.

Data analysis: The reproducible bands on gels were scored as present or absent for all accessions studied. The size of the fragments was determined both visually and using a *Pharmacia* (Piscataway, USA) laser densitometer equipped with gel scan (*GSXL*) software. A pairwise difference matrix and the phylogram tree between accessions were determined with the microcomputer program *PAUP* (*Phylogenetic Analysis Using Parsimony*, version 3.1) developed by D.L. Swofford (Illinois Natural History Survey, Champaign, IL 61820, USA).

Results

Fragment identification: The size of amplified DNA fragments ranged from 150 to 2300 bp. The number of bands in the profiles varied, depending on the primer and species tested. The number of bands ranged from 1 (*H. spontaneum*, primers F01, F09) to 10 in lanes 3 and 4 (*H. bogdanii*, primers E01, E02) (Figs. 1, 2). A total of 46 products that could be used as potential genetic markers were disclosed by the four primers. These fragments could be classified into three categories with respect to their polymorphism. Among them 10 fragments were common in both wild and cultivated *Hordeum* species, 23 fragments were specific to wild species and 3 fragments were specific to cultivated species.

Accession relationships: The pairwise distances among all the accessions computed by the PAUP program could be used to quantify the differences among them (Tables 2, 3). These data were based on the number of fragments which were different between each pair of cultivars. In spite of the wide range in the differences between the species, a smaller trend could be observed within the species. Thus

RAPDs clearly separated *H. bogdani, H. bulbosum, H. californicum, H. muticum, H. procerum, H. spontaneum* and *H. vulgare* species (a phylogram tree, Fig. 3).

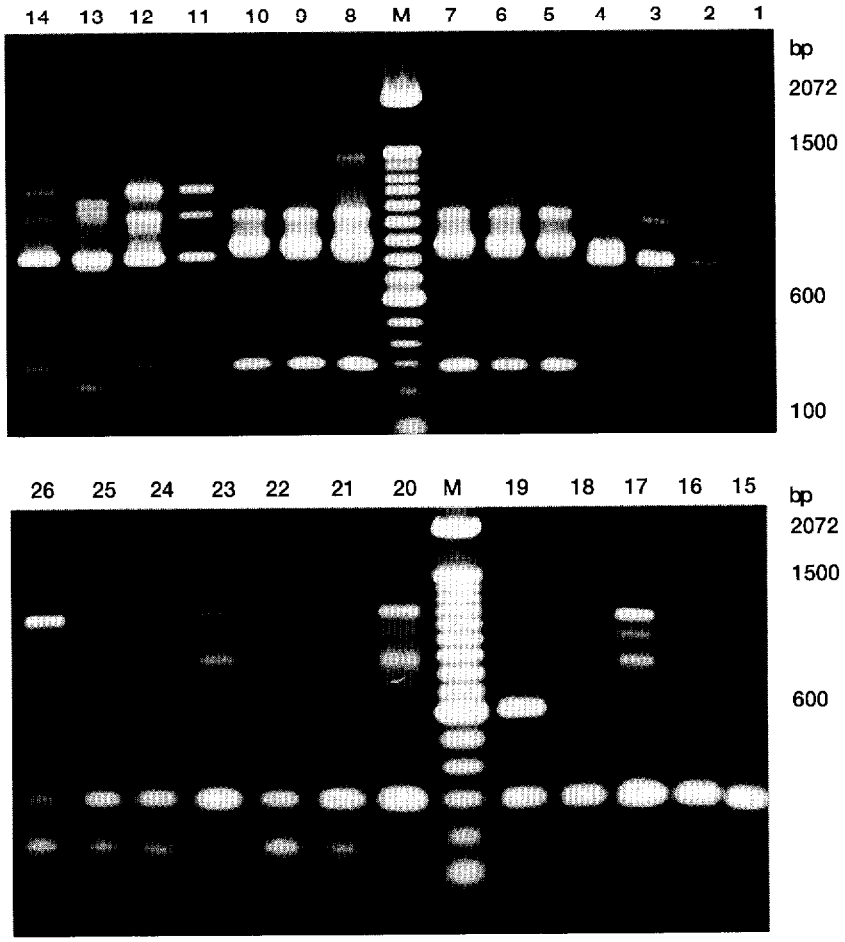

Fig. 1. RAPD profiles generated by primers E01 (*above*) and E02. Numbers on top refer to the accessions listed on Table 1. *M* indicates the 100 bp molecular size standard.

Discussion

We found polymorphic RAPD fragments both within and among populations of wild and cultivated barley. Although the single primer RAPD assay has great potential in detecting polymorphisms, the two primer assay (Klein-Laankhorst *et al.* 1991)

followed here also produces the polymorphisms that can be detected with a given set of random primers. The use of a combination of two primers resulted in the generation of a new specific fingerprint that was not merely the composite of the two fingerprints produced by the primers separately.

Of the two two-primer fingerprints analyzed for polymorphisms between wild and cultivated species, three cultivated species specific fragments (E1,2-550; E1,2-750 and F1,9-350) were obtained. Twenty-three wild species specific markers were also

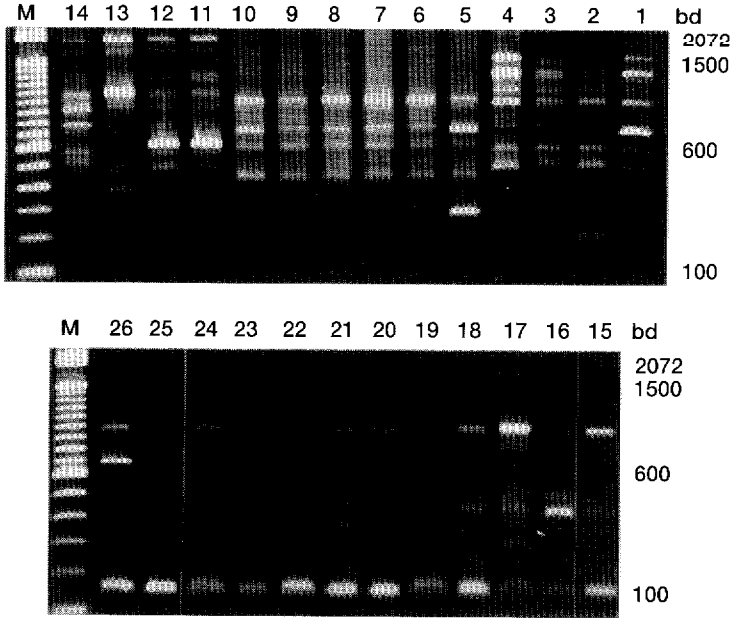

Fig. 2. RAPD profiles generated by primers F01 (*above*) and F09. Numbers on top refer to the accessions listed on Table 1. *M* indicates the 100 bp molecular size standard.

revealed by these two primer sets. Our RAPD analyses have implications for the systematics of *Hordeum*. From our RAPD data, a phylogram based on fragment differences places *H. vulgare* and *H. spontaneum* close to each other (Fig. 3). This supports the well-accepted hypothesis that the latter is the wild progenitor of the former (Kataoka *et al.* 1987). This same conclusion is derived from the RFLP data (Clegg *et al.* 1984, Neale *et al.* 1986). Clegg *et al.* (1984) concluded that variability is lower in cultivated barley than in wild barley, possibly as a result of domestication. In contrast, Holwerda *et al.* (1986) concluded that there were no differences in DNA diversity between cultivated and wild barley. Our results based on random primer pairs support the conclusion of Clegg *et al.* (1984) that wild barley is more variable

than cultivated barley. The average marker difference among wild barley (*H. spontaneum*) was 3.0, but 2.3 between cultivated barley (*H. vulgare*) (Table 3).

Fig. 3 also shows that *H. bulbosum* is close to *H. vulgare*. This agrees with the Wagner parsimony phylogenetic tree constructed by Doebley *et al.* (1992). Their study shows that *H. vulgare* and *H. bulbosum* constitute a monophyletic group distinctly differentiated from all other *Hordeum* species. This group was distinguished from all other *Hordeum* species by 11 synapomorphies. Both *H. vulgare* and *H. bulbosum* are of southwestern Asiatic-Mediterranean origin and their differentiation from other species was probably an ancient event. The two species are, however, clearly distinct from one another. Cytogenetic evidence showed that *H. vulgare* and *H. bulbosum* share a common basic genome, designated "I", which is somewhat differentiated between the two species (Kasha and Sadasivaiah 1971, Bothmer *et al.* 1983, Wang 1989).

Table 2. Pairwise marker difference among *Hordeum* accessions.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.
1.	-																									
2.	3	-																								
3.	8	5	-																							
4.	10	7	4	-																						
5.	18	19	22	20	-																					
6.	17	16	21	19	3	-																				
7.	17	16	21	19	3	0	-																			
8.	17	16	21	19	3	0	0	-																		
9.	17	16	21	19	3	0	0	0	-																	
10.	17	16	21	19	3	0	0	0	0	-																
11.	18	19	24	22	18	15	15	15	15	15	-															
12.	19	20	23	23	19	16	16	16	16	16	1	-														
13.	14	15	20	20	20	17	17	17	17	17	12	13	-													
14.	18	19	24	24	20	17	17	17	17	17	8	9	14	-												
15.	16	15	20	18	14	11	11	11	11	11	20	21	20	18	-											
16.	17	16	21	19	15	12	12	12	12	12	21	22	21	19	1	-										
17.	18	17	22	20	16	13	13	13	13	13	22	23	22	20	2	3	-									
18.	16	15	20	18	14	11	11	11	11	11	20	21	20	18	0	1	2	-								
19.	17	16	21	21	17	14	14	14	14	14	19	20	17	17	5	4	7	5	-							
20.	20	19	24	24	14	11	11	11	11	11	22	23	24	20	8	7	8	8	9	-						
21.	20	19	24	24	14	11	11	11	11	11	24	25	24	22	10	9	10	10	11	2	-					
22.	20	19	24	24	14	11	11	11	11	11	24	25	24	22	10	9	10	10	11	2	0	-				
23.	22	21	26	26	14	11	11	11	11	11	22	23	24	20	10	9	10	10	11	2	2	2	-			
24.	18	17	22	22	14	11	11	11	11	11	22	23	22	20	8	7	8	8	9	4	2	2	4	-		
25.	22	21	26	26	14	11	11	11	11	11	22	23	24	20	10	9	10	10	11	2	2	2	0	4	-	
26.	19	18	23	23	13	10	10	10	10	10	21	22	23	19	7	6	7	7	8	1	3	3	3	3	3	-

Our data indicate that the diploid, central Asiatic species, *H. bogdanii*, is more distantly related to species from North and South America. This agrees with the chloroplast DNA studies of *Hordeum* (Doebley *et al.* 1992) supporting earlier

Table 3. Average pairwise fragment difference among 7 *Hordeum* species.

Species	1.	2.	3.	4.	5.	6.	7.
1. *H. bogdannii*	6.2						
2. *H. bulbosum*	18.5	1.0					
3. *H. californicum*	21.0	16.0	1.0				
4. *H. muticum*	17.3	17.5	12.5	0.0			
5. *H. procerum*	21.3	17.5	8.5	14.0	0 0		
6. *H. spontaneum*	18.2	12.7	20.9	20.0	18.4	3.0	
7. *H. vulgare*	21.9	11.4	22.9	23.6	20.4	9.0	2.3

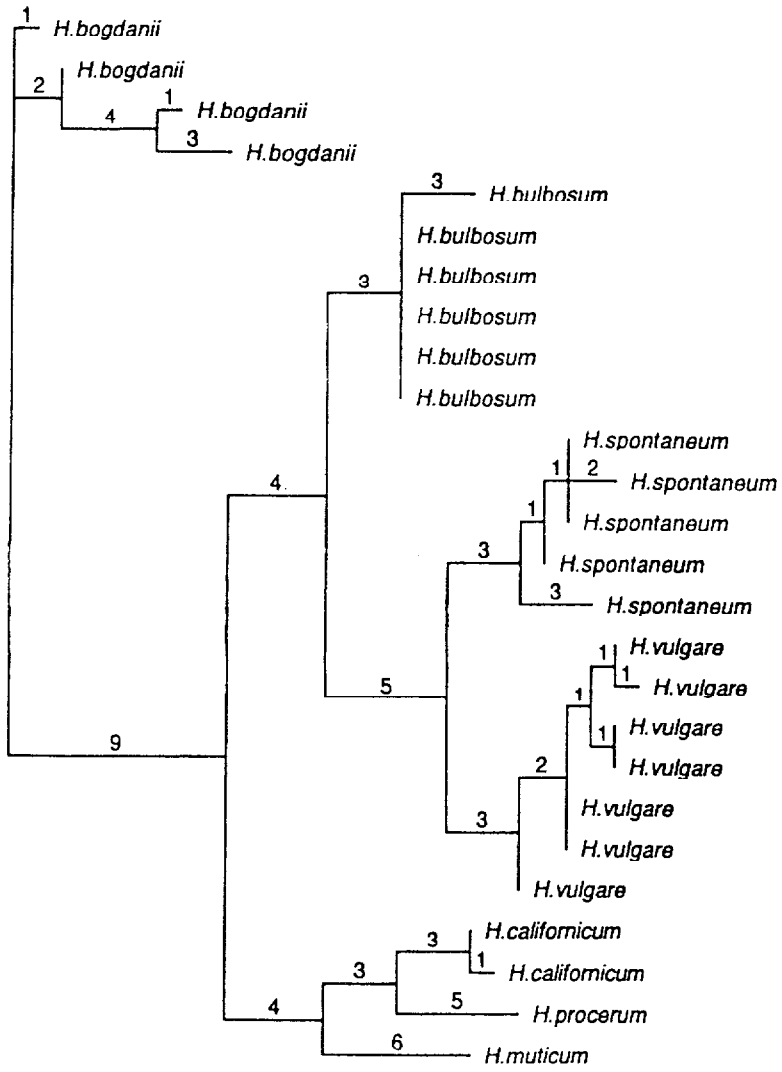

Fig. 3. The phylogenetic tree generated by the *PAUP* program. Branch lengths are indicated above branches.

evaluations concerning the distinctiveness of Asiatic members of the 9 genus based on crossing data, genomic relationships, and karyological data (Linde-Laursen *et al.* 1980, Bothmer and Jacobsen 1986).

From our RAPD based phylogeny, the diploid *H. muticum* and the hexaploid *H. procerum* form a monophyletic group (Fig. 3). Thus *H. muticum* may have been one of the ancestors of *H. procerum*. Previous cytological data indicated that *H. procerum* is not closely related to other South American hexaploid members of the genus (Subrahmanyam 1977).

In summary, two-primer RAPD fragments provided enough polymorphisms to construct a systematic phylogeny for *Hordeum*. Although a large number of 10-mer primers are commercially available, only four were necessary to separate the seven species studied. The data also were consistent with previously published isoenzymatic, molecular, and cytological data. Thus, the extensive polymorphism present in the *Hordeum* species and the speed of the method make feasible the use of the two-primer RAPD technique as an efficient tool for germplasm analysis and characterization.

References

Bonierbale, M.W., Paisted, R.L., Tanksley, S.D.: RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. - Genetics **120**: 1095-1103, 1988.

Bothmer, R.V. : Revision of the Asiatic taxa of *Hordeum* sect. *Stenostachys*. - Bot. Tidsskr. **74**: 117-147, 1979.

Bothmer, R.V., Jacobsen, N.: Interspecific crosses in *Hordeum* (*Poaceae*). - Plant Syst. Evol. **153**: 49-64, 1986.

Bothmer, R.V., Flink, J., Jacobsen, N., Kotimaki, M., Landstorm, T.: Interspecific hybridization with cultivated barley (*Hordeum vulgare* L.). - Hereditas **99**: 219-244, 1983.

Bothmer, R.V., Flink, J., Jacobsen, N., Jorgensen, R.B.: Variation and differentiation in *Hordeum marinum*. - Nordic J. Bot. **9**: 1-10, 1989a.

Bothmer, R.V., Flink, J., Landstorm, T.: Meiosis in interspecific *Hordeum* hybrids. I. Diploid hybrids. - Can. J. Genet. Cytol. **28**: 525-535, 1986.

Bothmer, R.V., Flink, J., Landstorm, T.: Meiosis in interspecific *Hordeum* hybrids. VI. Hexaploid hybrids. - Evol. Trends Plants **3**: 53-58, 1989b.

Clegg, M.T., Brown, A.H.D.: Whitfeld PR chloroplast DNA diversity in wild and cultivated barley: implications for genetic conservation. - Genet. Res. Cambridge **43**: 339-343, 1984.

Doebley, J., Bothmer, R.V., Larson, S.: Chloroplast DNA variation and the phylogeny of *Hordeum* (*Poaceae*). - Amer. J. Bot. **79**: 576-584, 1992.

Gupta, P.K., Fedak, G. , Molnar, S.J., Wheatcroft, R.: Distribution of *Secale cereale* DNA repeat sequence among 25 *Hordeum* species. - Genome **32**: 383-388, 1989.

Holwerda, B.C., Jana, S., Crosby, W.L.: Chloroplast and mitochondrial DNA variation in *Hordeum vulgare* and *Hordeum spontaneum*. - Genetics **114**: 1271-1291, 1986.

Jorgenson, R.B.: Biosystematics of *Hordeum bulbosum* L. - Nordic J. Bot. **2**: 421-434, 1982.

Jorgenson, R.B.: Relationships in the barley genus (*Hordeum*): an electrophoretic examination of proteins. - Hereditas **104**: 273-291, 1986.

Kasha, K.J., Sadasivaiah, R.S.: Genome relationships between *Hordeum vulgare* L. and *H. bulbosum* L. - Chromosoma **35**: 264-287, 1971.

Kataoka, J., Ogihara, Y., Tsunewaki, K.: Chloroplast DNA variation observed among *Hordeum* species. - Barley Genet. **5**: 515-524, 1987.

Klein-Lankhorst, R.M., Rietveld, P., Machiels, B., Verkerk, R., Weide, R., Koornneef, M., Zabel, P.: RFLP markers linked to the root knot nematode resistance gene Mi in tomato. - Theor. appl. Genet. **81**: 661-667, 1991.

Linde-Laursen, I.R., Bothmer, R., Jacobsen, N.: Giemsa C-banding in Asiatic taxa of *Hordeum* section *Stenostachys* with notes on chromosome morphology. - Hereditas **93**: 235-254, 1980.

Murray, M.G., Thompson, W.F.: Rapid isolation of high molecular weight plant DNA. - Nucl. Acids Res. **8**: 4321-4325, 1980.

Neale, D.B., Saghai Maroof, M.A., Allard, R.W., Zhang, Q., Jorgenson, R.A.: Chloroplast DNA diversity in populations of wild and cultivated barley. - Genetics **120**: 1105- 1110, 1986.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A., Allard, R.W.: Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. - Proc. nat. Acad. Sci. USA **81**: 8014-8018, 1984.

Slocum, M.K., Figdore, S.S., Kennard, W.C., Suzuki, Y.J., Osborn, T.C.: Linkage arrangement of restriction fragment length polymorphism loci in *Brassica oleracea*. Theor. appl. Genet. **80**: 57-64, 1990.

Subrahmanyam, N.C.: Haploidy from *Hordeum* interspecific crosses. I. Polyhaploids from *H. parodii* and *H. procerum*. - Theor. appl. Genet. **49**: 209-217, 1977.

Swofford, D.L.: PAUP: Phylogenetic Analysis Using Parsimony (version 3.1 computer program). - Illinois Natural History Survey, Champaign 1993.

Tanksley, S.D., Young, N.D., Paterson, A.H., Bonierbale, M.W.: RFLP mapping in plant breeding: new tools for an old science. - Bio/Technology **7**: 257-264, 1989.

Wang, R.C.: An assessment of genome analysis based on chromosome pairing in hybrids of perennial *Triticeae*. - Genome **32**: 179-189, 1989.

Welsh, J., McClelland, M.: Fingerprinting genomes using PCR with arbitrary primers. - Nucl. Acids Res. **18**: 7213-7218, 1990.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingley, S.V.: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. - Nucl. Acids Res. **18**: 6531-6535, 1990.

Wrigley, C.W., Batey, I.L., Skerritt, J.H.: Complementing traditional methods of identifying cereal varieties with novel procedures. - Seed Sci. Technol. **15**: 679-688, 1987.