Speciation and Species Separation in *Hordeum* L. (Poaceae) Resolved by Discontinuous Molecular Markers[1]

H. A. El-Rabey[1], A. Badr[2], R. Schäfer-Pregl[3], W. Martin[4], and F. Salamini[3]

[1] Genetic Engineering and Biotechnology Research Institute, Minufiya University, PO Box 79, Sadat City, Egypt
[2] Department of Botany, Faculty of Science, Tanta University, Tanta, Egypt
[3] Max Planck Institut für Züchtungsforschung, Köln, Germany
[4] Heinrich Heine Universität, Institut für Botanik III, Düsseldorf, Germany

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Abstract: **Amplified fragment length polymorphisms (AFLPs) were used to evaluate the capacity of discontinuous markers to reveal genetic structure within *Hordeum*, a challenging higher plant genus from the standpoint of natural systematics. Phylogenies of 63 accessions encompassing nine species from four *Hordeum* sections were inferred from polymorphisms scored at 600 loci. Phylogenies based on sequences from the nuclear internal transcribed spacer (ITS) region were constructed for comparison, but revealed severe sampling errors inherent to single genes. Although superior by virtue of providing genome-wide estimates of genetic similarity, the adoption of AFLPs in infrageneric studies requires caution. Comigrating AFLP bands studied here could be divided on the basis of band intensity variation into two types that are ~100% identical and <40% identical in DNA sequence, respectively, in infrageneric comparisons. Thus, the careful selection of AFLP bands to be analyzed bears heavily upon their phylogenetic utility. Within the *H. murinum* complex, which encompasses three morphologically distinct subspecies, AFLP data from 37 accessions reveal unexpected genetic differentiation between *H. murinum, glaucum* populations to the east and west of Alexandria (Egypt), suggesting the presence of allopatric speciation in the wake of human settlement.**

Key words: **AFLPs, phylogeny, speciation, hybridization.**

Abbreviations:
AFLPs: amplified fragment length polymorphisms
bp: base pair

Introduction

The genus *Hordeum* is one of the most challenging higher plant genera from the standpoints of evolution, taxonomy and natural systematics – one that exemplifies the problems surrounding the species concept in plants. It encompasses about 30 species dispersed in Eurasia, northern Africa, southern Africa, and Central and South America (Bothmer et al., 1995[12]). The most important member of the genus, *Hordeum vulgare* L. (barley), is one of the first crops domesticated by humans in the Middle East some 10 000 years ago. By comparing amplified fragment length polymorphisms (AFLPs) among 317 wild and 57 cultivated lines, the site of barley domestication was localized to a relatively small region spanning Israel and Jordan (Badr et al., 2000[4]). Yet beyond *H. vulgare*, available molecular, morphological or biogeographical data do not reveal a clear picture of the evolutionary history within this agronomically important genus.

Åberg (1940[1]) divided the genus into sections *Stenostachys* and *Bulbohordeum* (perennial) and *Campestria* and *Cerealia* (annual), whereas Nevski (1941[35]) recognized *Critesion, Stenostachys, Anisolepis, Hordeastrum, Bulbohordeum* and *Crithe*. Based on growth, phytogeography, ecology, cytology and cross compatibility, Bothmer and Jacobsen (1985[14]) included 31 species in the sections *Hordeum, Stenostachys, Anisolepis* and *Critesion*. Meiotic behaviour of interspecific hybrids (Bothmer et al., 1986[10], 1987[11]) suggests the existence of four basic genomes in the genus: H, I, X and Y. Among the species considered here, *H. vulgare* and *H. bulbosum* possess genome I, *H. murinum* possesses genome Y, *H. marinum* genome X and all remaining species genome H.

Bothmer et al. (1995[12]) stressed that cytogenetic and biochemical data suggest evolutionary hierarchies in *Hordeum* that differ from those implicit in morphological characters. These difficulties are compounded by dispersal patterns: for example, taxa of the *Hordeum murinum* complex are endemic in Europe, north Africa, southwestern Asia, the Caucasus, southern Uzbekistan, Tadzjikistan, Iran, and Afghanistan, but have also colonized most parts of the world as weeds (Bothmer et al., 1995[12]). *Hordeum* species also easily form interspecific hybrids, complicating reconstruction of the evolutionary history of the genus (Bothmer and Jacobsen, 1985[9]), and furthermore display complex patterns of shared characters across taxa. Such problems are commonplace in plant evolutionary studies at or near the poorly-defined species level (Bachmann, 1998[3]). And since allele-splitting often antedates speciation (Bachmann, 1998[3]), phylogenetic studies based on sequences of one or a few genes – as is currently common practice – does not offer an adequate solution to reconstructing evolutionary

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[1] Sequences of all sequenced AFLP bands discussed in this paper are available from the web site http://www.mpiz-koeln.mpg.de/~schaefer/hordeum/hord.html.

history within such genera (Avise, 1994 [2]; Nei and Kumar, 2000[31]; Graur and Li, 2000 [22]).

One approach to circumvent these problems in infrageneric studies in plants is to measure genome-wide genetic similarity using discontinuous markers. Among them, AFLPs are the most popular (Bachmann, 1998[3]; Heun et al., 1997[23]; Martin and Salamini, 2000[29]). Although they generate vast amounts of data in the form of shared bands, in the absence of DNA sequences for such bands it is difficult to assess whether comigrating bands are truly homologous in different species. And as the proportion of shared bands decreases, the uncertainty in band homology becomes increasingly severe.

Here we report on AFLP banding and ITS sequencing in the study of phylogeny and structure of natural diversity across 63 *Hordeum* accessions stemming from distinct geographical regions and representing all major sections of the genus. We also apply discontinuous markers to analyze the Mediterranean–Eurasian *Hordeum murinum* taxa, which exemplifies mosaic patterns of shared characters and problematic natural systematics. Finally, we address the sequence homology of comigrating AFLP bands in *Hordeum* in a case study, to examine the utility of AFLPs for plant natural systematics at the infrageneric level.

Materials and Methods

Plant material, character scoring and cytology

In experiment one, 63 accessions of nine *Hordeum* species were sampled from the Egyptian flora or supplied by gene banks. They represent the main *Hordeum* sections of Bothmer et al. (1995[12]). Accessions studied are given in Table **1** (exact sources of seeds and country of origin are given in the Supplementary Table on the website http://www.mpiz-koeln.mpg.de/salamini/~schaefer/mono/mono1.html). Identification of Egyptian materials was confirmed by the comparison to herbal sheets from the Royal Botanical Garden, Kew, U.K.

In experiment two, 37 accessions of the *H. murinum* complex were sampled from natural habitats in Egypt or supplied by gene banks (Table **1**). Morphological investigations were carried out on 10 plants per accession and twenty characters were scored. Codes were assigned to qualitative traits, while quantitative ones were measured (details in Table **3**). Cytological studies used the Feulgen technique and five metaphase plates per line were analyzed.

Genomic DNA extraction and AFLP analyses

Young leaves were collected, lyophilized and kept at – 70 °C until use. DNA was extracted according to the CTAB method (Saghai-Maroof et al., 1984[39]). AFLP fingerprinting was according to Zabeau and Vos (Zabeau and Vos, 1993[51]). Genomic DNA was restricted using EcoRI and MseI. The five primer combinations E37/M33, E37/M38, E41/M33, E41/M40, and E42/M38 were used in both experiments to generate AFLP fragments (Badr et al., 2000[4]). Fragments were separated on 4.5% polyacrylamide sequencing gels (Vos et al., 1995[48]). Hyperfilm-MP (Amersham, England) was exposed to the gels for 3 to 5 days. Polymorphic bands across all accessions in AFLP autoradiograms were scored generating a database of 600 AFLP fragments in the first experiment and 306 fragments in the second experiment.

Table 1 *Hordeum* accessions sampled

Experiment	Species/subspecies	*N*[a]	Abbr.
Experiment 1	*H. bulbosum*	9	bulb
	H. murinum glaucum	7	glau
	H. murinum leporinum	1	lepo
	H. murinum murinum	3	muri
	H. vulgare agriochriton	3	agri
	H. vulgare distichon	3	dist
	H. vulgare hexastichon	3	hexa
	H. vulgare spontaneum	7	spon
	H. chilense	2	chil
	H. pusillum	3	pusi
	H. jubatum	3	juba
	H. bogdanii	7	bogd
	H. brevisubulatum	5	brev
	H. marinum	7	mari
Experiment 2	*H. murinum glaucum*	18	glau
	H. murinum leporinum	8	lepo
	H. murinum murinum	11	muri

Complete information on exact sources of material investigated and geographical sites of seed sampling can be found in the Supplementary Table on the website http://www.mpiz-koeln.mpg.de/salamini/~schaefer/mono/mono1.html.

[a] Number of individual accessions studied.

Cloning and sequencing of AFLP fragments

Cutting AFLP fragments from gels introduces a degree of error, due to untraceable amounts of other fragments at the position excised from the gel. A second source of experimental error is the different ease with which specific fragments can be cloned. To clone AFLP fragments, fifty-nine of them common to different species were chosen. The fragments were excised from AFLP gels, dissolved in 100 µl distilled water, heated at 100 °C for 10 min, transferred to ice bath and centrifuged for 3 min at 10 000 rpm. Aliquots were amplified and the amplification products were electrophoresed on 2% agarose gel, excised under UV light and recovered using the QIAEX II kit (Qiagen). DNA fragments were ligated to the Promega-T Easy vector at 4 °C and transferred to 50 µl of competent cells. Transformation took place by heat shock for 45 sec at 42 °C. For DNA sequencing, plasmid mini preparations were made from transformed colonies using the Qiagen Plasmid Mini Kit. Sequencing was in the Applied Biosystem Prism 377 DNA sequencer (Perkin-Elmer). DNA sequences of fragments were compared using the tools of the Wisconsin package (GCG, version 9. 1).

Amplification of ITS DNA sequences

ITS sequences were amplified and sequenced as in Hsiao et al. (1993[24]). ITS2, ITS3, ITS4, and ITS5 primers were designed according to White et al. (1990[49]). The sequences were: ITS2, 5-GCT GCG TTC CTT CAT CGA TGC-3; ITS3, 5-GCA TCG ATG AAG AAC GCA GC-3; ITS4, 5-TCC TCC GCT TAT TGA TAT GC-3; ITS5, 5-GGA AGT AAA AGT CGT AAC AAG G-3. The amplified PCR products were separated on 1% agarose gel. The entire ITS

band was excised and recovered by using the QiaxII kit (Qiagen) and sequenced as described.

Phylogenetic analyses

Scores were assigned to individual AFLP fragments after having established the state of "common bands" to fragments of different species. In Fig. **1**, an AFLP autoradiogram is reproduced with bands considered common or not to particular taxa. Only bands belonging to type 1 (see Results) were scored for phylogeny. Scoring of morphological characters is described in Table **3**.

AFLP phylogenies were based on genetic distances calculated according to Dice (1945[16]) using the SAS package (1989). AFLP loci were scored as showing an allele-specific band (1) or not (0). In a pairwise comparison, two genotypes are similar ($1:1=a$; $0:0=d$) or different ($1:0=b$; $0:1=c$). The Dice genetic distance is $2a/(2a+b+c)$, that is, d is excluded while a double weight is assigned to a. The rationale is that the absence of an AFLP band can be due to different genetic reasons and, thus, it may not contribute to genetic similarity (see also Skroch et al., 1992[41]). AFLP-based trees were constructed by Neighbor-Joining (Saitou and Nei, 1987[40]) as implemented in PHYLIP (Felsenstein, 1993[18]) using the matrix of pairwise Dice distances. CONTML (PHYLIP) analyses of AFLP allele frequencies were performed using infraspecific (or infrasubspecific in the case of the *H. murinum* and *H. vulgare* complexes) frequencies for each AFLP band as calculated with SAS, yielding 600 frequency values for each of 14 OTUs (operational taxonomical units). Trees inferred from morphological characters were generated with CONTML of PHYLIP. ITS trees were inferred from the entire ITS region, including the 5.8 S DNA, using programs of the PHYLIP package. Neighbor-Joining trees were based on Kimura distances. Maximum likelihood ITS trees were constructed using a transition/transversio ratio of 2 and base frequencies estimated from the data.

Results

The degree of sequence identity among comigrating AFLP fragments of different species

At the infraspecific level, discontinuous markers, such as AFLPs, are powerful tools and the homology of bands can easily be tested across genotypes in segregating populations. In large interspecific comparisons, however, the allelic nature of AFLP fragments is more tedious to assess – only sequencing can establish that two comigrating fragments are homologous. The problem is not trivial because regardless of what algorithm is used to calculate a genetic distance based on markers, the starting point is the number of comigrating DNA fragments shared (or not shared) by two taxa. In essence, it has to be established that comigrating AFLP fragments are homologous across species, and large-scale studies in plants for comparison have not been reported.

In their lower regions, sequencing gels used in AFLP analysis distinguish bands at a resolution of one nucleotide. In higher positions, a difference becomes evident only at a resolution of two or even more nucleotides. In addition to length, band intensity differences are also observed (Fig. **1**). We cloned and sequenced two distinguishable types of AFLP fragments to see

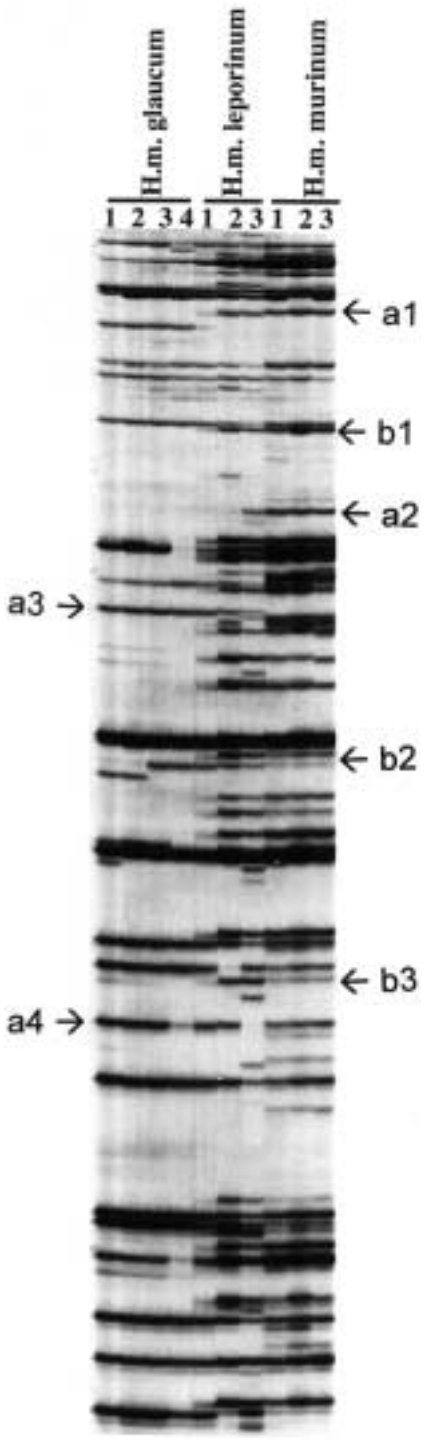

Fig. 1 Banding pattern produced by the AFLP primer combination E41/M40 of accessions of *H. m. glaucum, H. m. leporinum* and *H. m. murinum*. Type 1 and 2 fragments are respectively labeled with a and b. The assignment to fragments of the state of *common band* was as follows: a1: bands are considered *common* in *H.m.l.* 2, 3 and *H.m.m.*, 1, 2, 3. a2: bands were *common* in *H.m.l.* 3 and *H.m.m.* 1, 2, 3. a3: bands are *common* in *H.m.l.* 1, 2, 3 and *H.m.g.* 1, 2, 3. a4: bands are *common* in *H.m.l.* 1, 2, *H.m.g.* 1, 2, 3 and *H.m.m.* 1, 2, 3. b1: bands are common only within taxa. b2: bands are different even within taxa. b3: bands of difficult definition.

Table 2 Sequence identity in *Hordeum* AFLP fragments

Fragment		N_{spp} [a]	N_{band} [b]	Size (bp)	Id_{sp} [c] (%)	Id_{gen} [d] (%)
Type 1 [e]	A	5	11	290 – 291	100	82.1 – 100
	B	3	5	143 – 144	100	99.3 – 100
	C	3	6	121	100	85.1 – 96.7
		2	2	121	n.d.[g]	< 40, < 40
	D	2	4	110	100	100
	E1	2	7	271	100	98.2
	E2	2	2	268 – 269	n.d.	98.0
Type 2 [f]	F	3	12	129	100	92.9 – 100
		2	2	128	n.d.	99.2, < 40
		1	1	128	n.d.	< 40
	G	2	2	113	n.d.	< 40
		2	2	111	n.d.	100
	H	2	2	284	n.d.	< 40
	I	2	2	380	n.d.	< 40

[a] Number of species with common fragment; [b] No. of bands cloned and sequenced; [c] Infraspecific sequence identity; [d] Infrageneric sequence identity; [e] The AFLP fragments migrated at the same position and had similar intensities; [f] The AFLP fragments migrated at the same position but had different intensities; [g] not determined.

how much sequence identity they share. The first type encompassed bands that were identical across species in both signal intensity and migration position (type 1; Fig. **1**, a). The second group encompassed fragments with borderline identity of migration position (that is, apparently less than one nucleotide difference in comparisons) and evident differences in band intensity (type 2; Fig. **1**, b).

Type 1 AFLP fragments are designated as A, B, C, D, E1 and E2 in Table **2**. For fragment A, five sequences from *H. bogdanii* were 290 or 291 bp long, two sequences were produced from *H. brevisubulatum* (291 bp), two from *H. chilense* (292 bp), one from *H. pusillum* (291 bp) and two from *H. jubatum* (291 and 290 bp). Intraspecific sequence identity of all these bands was always 100%. Interspecific identities varied between 82.1 and 100%. The 100% identity of the *H. jubatum* and *H. bogdanii* fragments with 290 and 291 bp merely indicates that cloning procedures can entail loss of a base. Fragment B was cloned in *H. chilense* (143 bp), *H. pusillum* (144 bp) and *H. jubatum* (143 bp) and sequenced 2, 1, and 2 times, respectively. Sequence identity was 100% within species and from 99.3% to 100% across species. Fragment C (121 bp long) was cloned from *H. bulbosum* (three sequences), *H. brevisubulatum* (one), *H. marinum* (one), *H. chilense* (one) and *H. pusillum* (two). Within species, sequence identity was 100%. Among *H. bulbosum*, *H. chilense* and *H. pusillum*, sequence identity was from 85.1 to 96.7%. *H. marinum* and *H. brevisubulatum* had fragments without significant similarity to each other or to fragments from other species. Fragment D was sequenced (twice) both in *H. brevisubulatum* (110 bp) and *H. chilense* (110 bp). Intra- and interspecific sequence identity was 100%. Fragments E1 and E2 had different, although close, migrating positions. E1 (271 bp) was cloned from *H. vulgare* (4 sequences) and *H. procerum* (3). The sequence identity of this fragment between species was 98.2%. E2 was cloned from *H. bogdanii* (269 bp; 1 sequence) and *H. chilense* (268 bp; 1 sequence). The homology between the 2 versions was 97%.

Table 3 Characters distinguishing taxa within *H. murinum* complex

Character	Taxon		
	H. m. glaucum	*H. m. leporinum*	*H. m. murinum*
Culm length (cm)	43 ± 0.9	63.0 ± 1.9	71.0 ± 0.8
Comparative culm thickness	slender	intermediate	intermediate
Flag leaf length (cm)	6.19 ± 0.38	9.50 ± 0.57	16.9 ± 0.61
Flag leaf width (cm)	0.59 ± 0.03	0.74 ± 0.06	1.22 ± 0.01
Comparative auricle size	small	intermediate	intermediate
Peduncle length (cm)	18.6 ± 0.84	13.8 ± 0.93	21.2 ± 0.55
Relative time of flowering	early	intermediate	late
Spike length (cm)	5.17 ± 0.10	9.50 ± 0.19	8.5 ± 0.41
Spike breadth (cm)	0.73 ± 0.01	1.09 ± 0.06	0.92 ± 0.01
Rachis length (cm)	0.20 ± 0.00	0.30 ± 0.00	0.30 ± 0.01
Central spikelets	pedicellate	pedicellate	sessile
Central vs. lateral spikelets	shorter	shorter	equal
Central spikelet anther length	0.05 ± 0.00	0.13 ± 0.00	0.10 ± 0.00
Central spikelet anther color	red spotted yellow	yellow	yellow
Central spikelet awn length (cm)	3.58 ± 0.11	5.10 ± 0.36	4.10 ± 0.28
Lateral spikelet awn length (cm)	3.39 ± 0.13	3.60 ± 0.33	3.60 ± 0.20
Lateral spikelet anther length (cm)	0.15 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
Lateral spikelet palea	pilose	scabrid	glabrous
Grain length (cm)	0.68 ± 0.01	1.00 ± 0.00	1.00 ± 0.02
Grain breadth (cm)	0.20 ± 0.00	0.30 ± 0.00	0.22 ± 0.01

The data presented are mean values of 18, 8 and 11 accessions respectively for *H. m. glaucum*, *H. m. leporinum* and *H. m. murinum* (SE is provided for quantitative values). In tree building, for characters 2, 5, 11, 12 and 14 the codes 1 and 2 were assigend to the two alternative states. For three state characters (7 and 18) the codes were 1, 2 and 3.

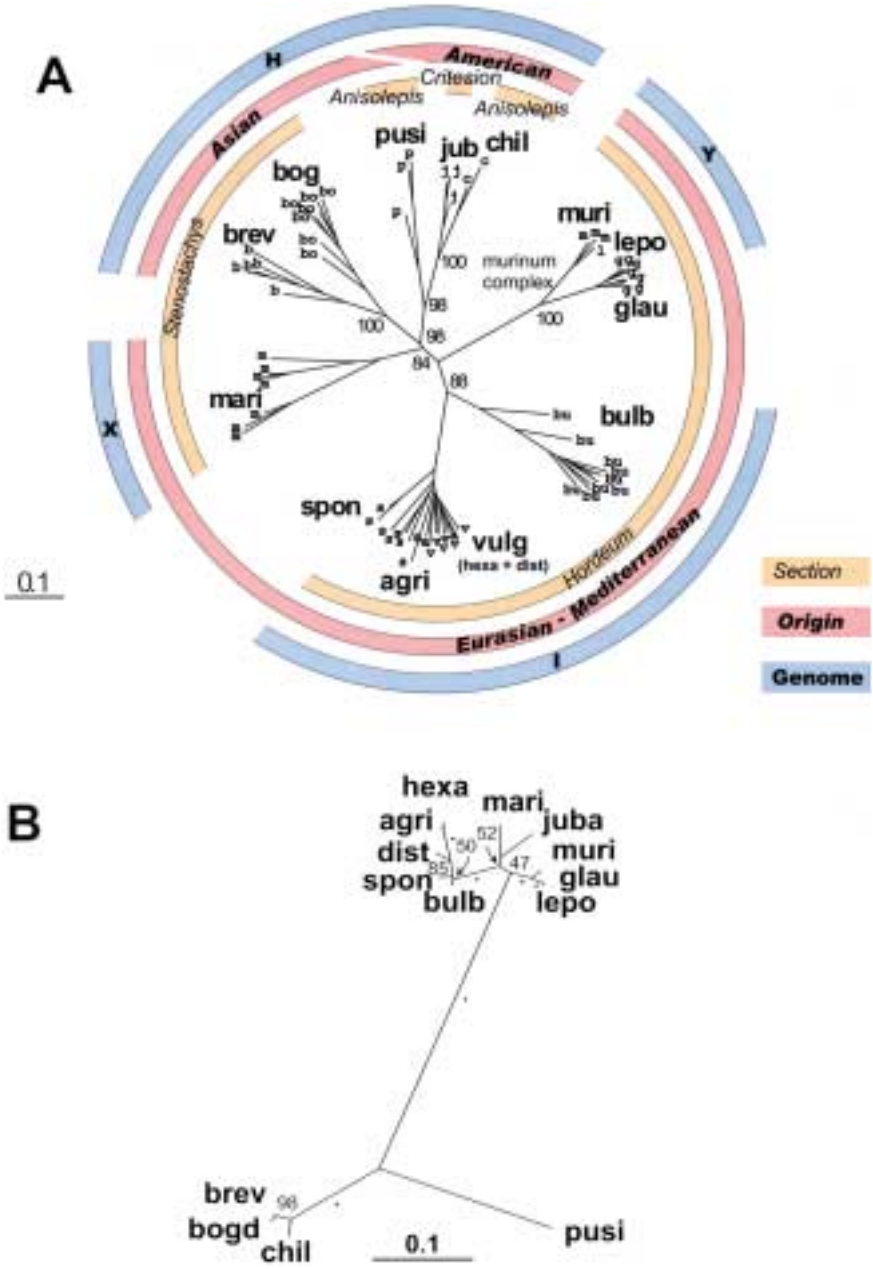

Fig. 2 (**A**) Tree constructed from AFLP data (5 primer combinations, 600 fragments) encompassing 63 *Hordeum* accessions from 14 species and subspecies covering 4 sections (see Table **1**). Abbreviations are from the species names given in Table **1**. The Neighbor-joining tree was based on Dice distances. Numbers indicate the bootstrap proportions (BP) among 100 trees inferred from infraspecific and infrasubspecific allele frequencies using CONTML of PHYLIP (see Methods). (**B**) Tree inferred from ITS sequences from the same 14 taxa. The complete region spanning ITS1 and ITS2 including the coding region of the 5.8 S rRNA gene (total 613 positions) was used for analysis. The figure shows the Neighbor-joining tree of Kimura distances as estimated with PHYLIP. Numbers at branches indicate the corresponding BPs from 100 replicates. Dots indicate branches with BP = 100. The scale bar indicates 0.1 substitutions per site. Using DNAML with a transition/transversion ratio of 2.0 and base frequencies estimated from the data, a similar overall topology was found, whereby no branches with BP ≥ 70% conflicted with the topology shown.

Type 2 AFLP fragments are designated in Table **2** as F, G, H and I. Fragment F (129 bp) was cloned four times from *H. vulgare, H. marinum* and *H. procerum. H. bogdanii, H. bulbosum* and *H. brevisubulatum* had F fragments of 128 bp (one sequence per species). Sequences of the 129 bp version were 100% identical within species and 82.9 to 100% identical between species. Sequences of the 128 bp version were 99.2% identical between *H. bogdanii* and *H. brevisubulatum,* while in *H. bulbosum* it was unrelated to other sequences. The 129 bp and 128 bp versions of fragment F shared less than 40% identity. Fragment G was cloned from *H. bogdanii* and *H. brevisubulatum* (111 bp, one sequence) and from *H. murinum* and *H. bulbosum* (113 bp, one sequence). The 113 bp version had less than 40% identity between species, while the two 111 bp versions were 100% identical. Fragment H (284 bp) was sequenced from *H. murinum* (one) and *H. jubatum* (one), but these two sequences were unrelated. The case of F, G and H bands was clear: different species may have or not have homologous comigrating fragments. Sequences of all bands mentioned are available from the web site http://www.mpiz-koeln.mpg.de/~schaefer/hordeum/hord.html.

AFLP band sequencing supports the assignment of a high phylogenetic value to type 1 fragments. Type 2 fragments also have informative content, but they can be considered in phylogenetic studies only if associated with a majority of the first type of fragments. Distinction between a type 1 and a type 2 fragment is a matter of phenotype scoring and in many cases is not simple.

Infrageneric Hordeum *phylogeny inferred from AFLPs*

AFLP phylogenies were based on 600 band positions common to at least two species (monomorphic bands excluded). To avoid type 2 bands, the first upper 8 cm of the AFLP autoradiograms (fragments > 400 bp long) were not scanned. Nevertheless, it cannot be excluded that type 2 fragments contributed to a minor fraction of our database; but all practical caution was taken to purge the data of suspectedly non-homologous bands. Of the 600 bands scored, 7 were constant and 9 were autapomorphic.

Data were analyzed with different methods of genetic distance calculation and tree building, giving results as in Fig. **2A**. With a minor exception, species clustered in full concordance with geographic origin, cytology and taxonomical status: the Mediterranean–Eurasian species – the *H. murinum* complex (genome Y), *H. vulgare* and *H. bulbosum* (genome I) – were well separated, as well as *H. bulbosum* from *H. vulgare*. The three Asian species with genome H, *H. bogdanii*, *H. brevisubulatum* and *H. pusillum* (that is also American) clustered near to the two American species with genome H (*H. chilense* and *H. jubatum*). *H. marinum* (genome X) positioned between Asian species and *H. vulgare*.

Comparison of AFLP and ITS phylogenies

The ITS region was amplified and sequenced in one accession per species. The properties of ITS regions showing the length of ITS 1, 5.8S and ITS 2, their base frequency, adenine–thymine (AT) and guanine–cytosine (GC) ratio as well as ITS sequences are deposited at the web site http://www.mpiz-koeln.mpg.de/~schaefer/hordeum/hord.html. The nucleotide sequence of ITS 1 revealed conspicuous indels such as a 14 bp deletion in *H. chilense, H. pusillum, H. bogdanii* and *H. brevisubulatum*. The sequence of ITS 2 appeared more homogenous. ITS phylogeny was less powerful than the one derived from AFLPs (Fig. **2B**), for example the *H. vulgare* complex included *H. bulbosum*, a species morphologically and physiologically distinct. The *H. murinum* complex was well-separated. Two clusters inferred from ITS sequences were problematic: *H. marinum* (Mediterranean–Eurasian; genome X) appeared tightly related to *H. jubatum* (American; genome H); the same was evident for the cluster *H. pusillum, H. chilense, H. bogdanii* and *H. brevisubulatum*, all species with genome H, but one of American origin and the other three Asiatic or American–Asiatic. ITS from *H. jubatum* (American) was far removed from the latter cluster and lacked the 14 bp deletion in ITS 1 in *H. pusillum, H. chilense, H. bogdanii* and *H. brevisubulatum*.

Phylogenetic dissection of the H. murinum *complex*

The *H. murinum* complex contains 3 taxa of ambiguous status: they have been recognized as independent species (Bor, 1970[8]; Täckholm, 1974[45]; Baum and Bailey, 1984[5], 1984[6]), but also as conspecific subspecies or morphotypes (Giles, 1984[20]; Giles and Lefkovitch, 1986[21]; Bothmer et al., 1995[12]). Using AFLPs, 18 *H. m. glaucum*, 8 *H. m. leporinum* and 11 *H. m. murinum* accessions were studied. These were either sampled in Egypt, or obtained from seed banks. Taxa assignment was based on samples from Kew Botanical Gardens. The taxa are morphologically easily distinguishable, even if their characters are distributed in a mosaic across the complex.

Table **3** summarizes average values of taxonomically relevant morphological traits. A phylogenetic tree based on morphological data is presented in Fig. **3A**. The three taxa are well separated and two details emerge: accession L8 (the only hexaploid studied) has a topology intermediate between *H. m. leporinum* and *H. m. murinum*; accessions M1 and M2 cluster with the *H. m. murinum* group but are genetically more separated. The cytological results (Table **1**) confirmed previous reports: the *H. m. glaucum* accessions were diploid (2n = 2x = 14); the *H. m. leporinum* lines were tetraploid (2n = 28) although one hexaploid (2n = 42) was found (L8); all members of subsp. *murinum* were tetraploid.

The *H. murinum* complex was dissected by AFLP fingerprinting. Five primer combinations generated 306 polymorphic fragment positions (monomorphic bands were excluded). The data were used to calculate genetic distances and phylogenetic trees (Fig. **3B**). The polyploid taxa *H. m. leporinum* and *H. m. murinum* are closely related, while *H. m. glaucum* is well separated. *H. m. glaucum* falls into two clusters representing lines sampled in locations either to the west or to the east of Alexandria (Sinai desert included).

Discussion

Innan et al. (1999[25]) have estimated nucleotide diversity (π) (Nei, 1987[32]) from AFLP data. Their method considers nucleotide substitution in the cutting sites of restriction enzymes. Because similar AFLP fragments from different genotypes of cultivated barley map to the same genetic loci (Qi et al., 1997[37]; Castiglioni et al., 1998[14]), in this species the method of Innan et al. (1999[25]) leads to reliable estimates of π. In infrageneric comparisons, the same assumptions may not hold (Smith et al., 1994[42]; Spooner et al., 1991[43]). In fact, cutting sites flanking a given DNA fragment can be used in molecular taxonomy when the DNA they delimit is highly homologous across species. The problem is the definition of the level of DNA homology supporting interspecific synteny. Our experimental data help: cloned AFLP fragments, common to different species, have a high sequence identity (type 1: between 82.1 and 100%). The consideration of type 2 fragments, that show scorable differences across lanes and that share < 40% sequence identity, should be avoided. Thus, particular attention is required when choosing bands. Because type 1 AFLP fragments are an excellent indicator of sequence identity and, by inference, of chromosomal position AFLP-based phylogenies as generated here, they have an empirically robust molecular basis, at least in *Hordeum* (see also Rouppe van der Voort et al., 1997[38] on potato).

Previous analyses of six RFLP probes in *Hordeum* produced a tree of the genus coherent with cytological data, although it was maintained that *H. glaucum* was a subspecies of *H. murinum* (Svitashev et al., 1994[44]). By contrast, a single-gene topology (Komatsuda et al., 1999[27]) suggested a monophyletic relationship between American species and *H. marinum*, which conflicts with the brunt of available data. Sequence homology among some PCR amplified bands from different species was demonstrated, but only for one or few bands (Williams et al., 1993[50]; Tinker et al., 1993[47] ; Dos Santos et al., 1994[17]; Peltier et al., 1995[36]; Marillia and Scoles, 1996[28]). In spite of this there is a tendency in the *Hordeum* literature to claim the correspondence of molecular phylogenies with mor-

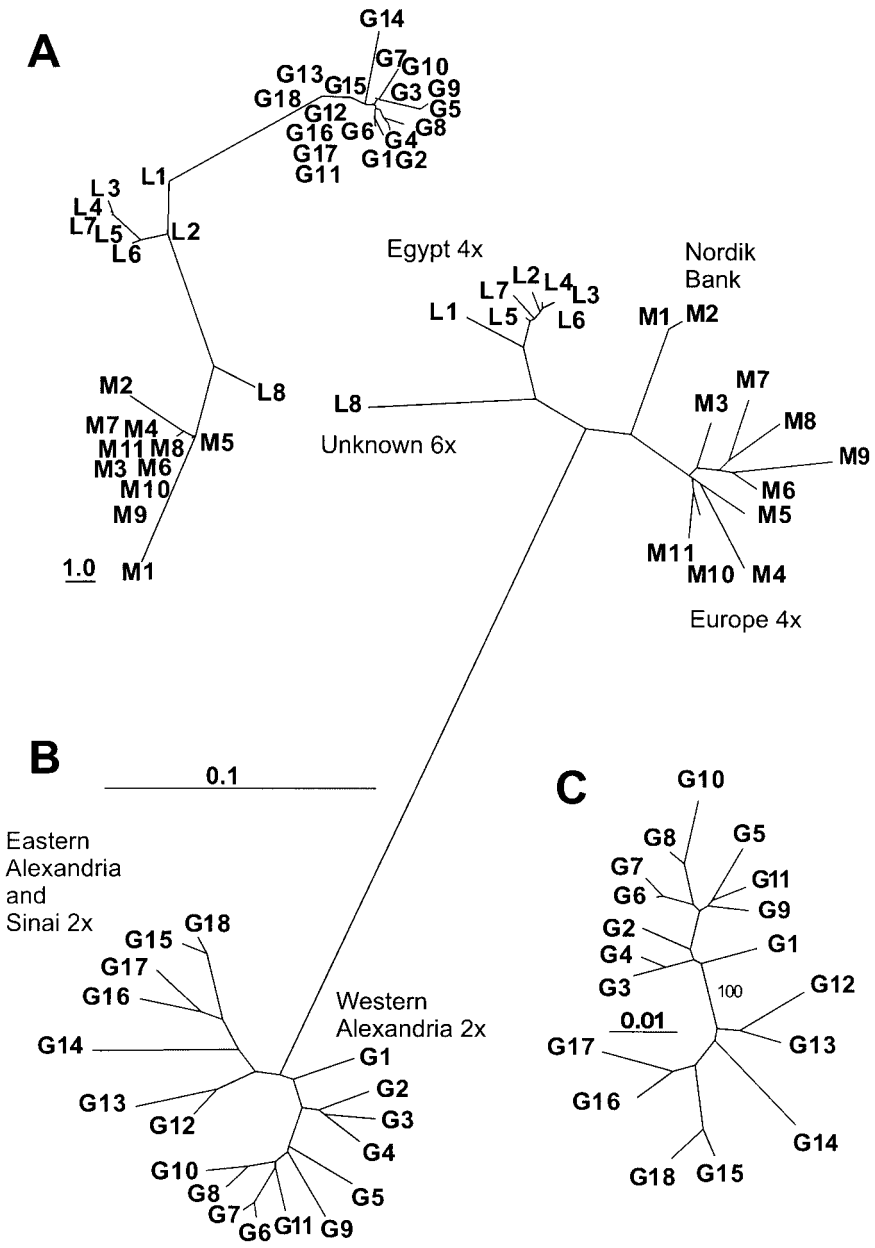

Fig. 3 (**A**) Maximum likelihood tree of continuous characters based on twenty morphological traits as listed in Table **2**. Scoring for quantitative or alternative state characters is described in Table **3**. The tree was constructed using CONTML in PHYLIP. (**B**) Neighbor-joining tree of Dice distances inferred from AFLP data (5 primer combinations, 306 fragments) considering 47 accessions of the *H. murinum* complex (listed in Table **1**, lower part). L = *H. m. leporinum*; M = *H. m. murinum*; G = *H. m. glaucum*. (**C**) Neighbor-joining tree of Dice distances from AFLP data for *H.m. glaucum* accessions only. The BP shown was determined from 100 trees inferred from allele frequencies using CONTML of PHYLIP for G1–G4, G5–G11, G12–G14 and G15–G18.

phology-based trees and cytological criteria. A careful consideration of published data, however, reveals substantial qualitative differences, that is, taxa occupy dramatically different positions across trees (Molnar et al., 1992[30]; De Bustos et al., 1996[15]; Marillia and Scoles, 1996[28]; Terzi et al., 2001[46]). This situation was also noted for the genus *Solanum* (Kardolus et al., 1998[26]). In addition, absolute genetic distances between taxa are usually not discussed. Overall, it appears that a comprehensive molecular phylogeny of *Hordeum* is still not available; but our results indicate that a survey of several accessions for each one of the known species, carried out with about 500 scorable AFLPs, would provide a hierarchy based on overall genome similarity that could be considered as conclusive.

The comparison between ITS sequence-based and AFLP-derived trees is clear: AFLPs produce topologies consistent with geographic origin, with cytological behaviour in crosses and for the most part with morphology. The tree based in ITS sequencing is at odds with such independent data. For example, the positioning in ITS topologies of *H. bulbosum* together with the *H. vulgare*, does not make biological sense either in terms of morphological traits such as vegetative reproduction or based on overall AFLP genetic distance. In addition, *H. jubatum* (American; H genome) appears to be the sister of *H. marinum* (Mediterranean; X genome) on the basis of ITS data, and this is biologically incorrect. The prime reason behind the superiority of AFLPs over ITS sequencing (or other single loci) at the infrageneric level is the large number of loci sampled (600 in the case of this data).

A further test of AFLP markers was in the discrimination of taxa within a species complex. The *H. murinum* complex is easily distinguished within the *Hordeum* section based on leaf blade characters (Bowden, 1962[13]). Within the complex, *H. m. murinum* shares more characters with *H. m. leporinum* than with *H. m. glaucum* (Table **3**, 1). *H. m. glaucum* is characterized by poor vigor, shorter spikes and the shorter red-spotted anthers of the central spikelet. *H. m. murinum* is unique in having sessile or sub-sessile central spikelets and in the glabrous palea of the lateral spikelet. The problem is a classical one: are we dealing with species or subspecies here? Giles (1984[20]) and Giles and Lefkovitch (1986[21]) considered three taxa with equal status. Based on the low fertility of their intercrosses, the three taxa could even be considered species, but subspecies when natural opportunities for crossing were considered. Bor (1970[8]), Täckholm (1974[45]) and Baum and Bailey (1984[5], 1984[6]) favor separate species. Our cytological and molecular investigations support the division of the complex into two groups with the rank of species: the diploid *H. glaucum* and the polyploid *H. murinum* including the subspecies *H. m. murinum* and *H. m. leporinum*.

Interestingly, two accessions of *H. m. murinum* obtained from the Nordic Gene Bank, designated as *H. m. murinum* 1 and 2 in Table **1** and as M1 and M2 in Fig. **3**, share both a combination of morphological characters distinctive from other M accessions and an intermediate position – as based on AFLP data – between *H. m. murinum* and *H. m. leporinum*. The finding of a continuum of lines between the two taxa is supported by results on chloroplast DNA restriction patterns (Baum and Bailey, 1989[7]) and *in situ* fluorescence hybridization (De Bustos et al., 1996[15]). It seems that M1 and M2 are either descendants of a yet unnammed taxon with the same (subspecies) rank as *H. m. murinum* and *H. m. leporinum*, or that the M1 and M2 are hybrids of *H. m. murinum* and *H. m. leporinum*.

Notably, the level of genetic diversity within *H. m. glaucum* accessions from the Mediterranean regions east and west of Alexandria (Egypt) is comparable to that found between *H. m. murinum* and *H. m. leporinum*, although no morphological differentiation is observable across these habitats. Among morphologically distinct grasses from geographically isolated populations, similar levels of genetic distinction as measured by dicontinuous markers have been found (Badr et al., 2000[4]; Heun et al., 1997[23]; Nevo, 1992[33]; Nevo et al., 1979[34]). The findings reported here indicate that allopatric *H. m. glaucum* populations to the east and west of Alexandria may have been separated due to human settlement in Egypt and may be in a state of incipient speciation.

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W. Martin

Heinrich Heine Universität
Institut für Botanik III
Universitätsstraße 1
40225 Düsseldorf
Germany

E-mail: w.martin@uni-duesseldorf.de

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