Species Relationships in *Hordeum*: Cytology and RAPD Analyses

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Received July 9, 2009; accepted August 25, 2009

Summary Cytological and molecular studies were performed on 10 *Hordeum* species of Iran. The species studied showed the occurrence of $2n=2x=14$, $2n=4x=28$ and $2n=6x=42$. They formed mainly bivalents in metaphase with normal chromosomes segregation during anaphase and telophase stages, except in few cases of laggard chromosomes formation and chromosome stickiness observed in *H. bulbosum*. *H. bulbosum* and *H. leporinum* showed 0–2 B-chromosomes which were smaller than A-chromosomes and did not pair with them out of 20 RAPD primers used 11 primers produced 18 reproducible polymorphic bands. Specific bands were observed in some of the species or populations while, some bands occurred in all the species except one showing the species genetic differences. Clustering of the species based on cytological and RAPD data partly produced similar results, separating *H. vulgare* and *H. spontaneum* from each other. The species of *H. marinum*, *H. glaucum* and *H. leporinum* from the sect. *Hordeastrum* were grouped together, while *H. violaceum*, *H. bogdanii* and *H. brevisubulatum*, from the sect. *Stenostachys* were placed in different clusters separated from each other. *H. bulbosum* and *H. vulgare* showed a close relationship, while *H. spontaneum*, *H. distichon* and *H. vulgare* were placed close to each other supporting their taxonomic position in Flora Iranica.

Key words Cytology, *Hordeum*, Iran, RAPD, Taxonomy.

The genus *Hordeum* L. (Pooideae) is considered among difficult plant groups from the standpoints of evolution and taxonomy, containing about 30 species growing in the North temperate and South America as well as in South-West and central Asia. They are annual or perennial taxa usually growing in open weedy or sandy places but mostly in dry soils (Bothmer *et al.* 1991, Shewry 1992, Watson and Dallwitz 1992), including weedy species like *H. jubatum*, *H. leporinum*, *H. marinum*, *H. murinum* and important grain crop species like Barley (*H. vulgare*). The *Hordeum* base chromosome number is $x=7$, and it contains about 40 diploid ($2n=2x=14$), tetraploid ($2n=4x=28$) and hexaploid ($2n=6x=42$) species (Bothmer *et al.* 1995). The chromosomes are large, with haploid nuclear DNA content 5.4–5.6 pg and mean diploid $2c$ DNA value of 11 pg. The species hybridize with species of other genera like *Elytrigia* ($\times$ *Elytrordeum* Hylander, $\times$ *Elyhordeum* Zizan & Petrowa), *Agropyron* ($\times$ *Agrohordeum* A. Camus), *Secale* ($\times$ *Hordale* Ciferri and Giacom.), *Sitanion* ($\times$ *Sitordeum* Bowden), *Triticum* ($\times$ *Tritordeum* Aschers. and Graebn.) (Watson and Dallwitz 1992).

Blattner (2004) suggested that the genus *Hordeum* originated about 12 MYA in western Eurasia then expanded its distribution to Europe, central Asia, North America, South America, and South Africa. A morphological study made by Seberg and Frederiksen (2001) suggested monophyly of the genus, which was supported by morphological and molecular phylogenetic studies of

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the latter researchers (Petersen and Seberg 1997, Blattner 2004). The classification of the genus *Hordeum* was initially based on morphological traits, later on cytogenetic traits, and the ability of species to hybridize and produce fertile progeny in crosses was used in systematics of the genus (Bothmer and Jacobsen 1985). Controversy exists about taxonomic treatment of the genus *Hordeum*; for example Åberg (1940) divided the genus into 4 sections of *Stenostachys*, *Bulbohordeum*, *Campestris* and *Cerealina* (the first 2 sections are perennial and the other 2 are annual) while Nevski (1941) divided the genus into 6 sections of *Critiesion*, *Stenostachys*, *Anisolepis*, *Hordeastrum*, *Bulbohordeum* and *Crithe*. Later on Bothmer and Jacobsen (1985), by considering the growth pattern, phytogeography, ecology, cytology and cross compatibility of the *Hordeum* species divided the species in 4 sections of *Hordeum*, *Stenostachys*, *Anisolepis* and *Critiesion*. Difficulties exist in the species morphological characters due to the presence of complex patterns of shared characters across taxa (Bachmann 1998). These problems have led to controversies about taxonomic status of several taxa within the genus. For instance, some researchers consider *H. geniculatum* All., as a separate species but other authors assign it to *H. marinum* Huds. *sensu lato*. Likewise, taxonomic relationships between *H. murinum* and *H. glaucum* are not completely established (Kochieva *et al.* 2001). The other point is that *Hordeum* species hybridize with each other further complicating reconstruction of the evolutionary history of the genus (Bothmer and Jacobsen 1985).

Extensive cytological studies have been carried out in the genus *Hordeum* mainly concerned with chromosome number and polyploidy level (Bothmer 1979, Bothmer *et al.* 1986a, b, 1987, Zhang *et al.* 1990, Jahan *et al.* 1992, Bothmer *et al.* 1995, Bao-Rong 1997, Sheidai *et al.* 2008). The study of the meiotic behaviour of interspecific hybrids suggested the existence of 4 basic genomes of H, I, X and Y in the genus (Bachmann 1998). *H. vulgare* (2x) and *H. bulbosum* (2x, 4x) have the I genome, *H. marinum* (2x, 4x) and *H. murinum* (2x, 4x, 6x) have distinct genomes of X and Y respectively and the rest of diploid species have different forms of H genome.

Biochemical and molecular markers including storage protein fraction of barley (hordein), isoenzymes, RAPD and inter-simple sequence repeats (ISSR), amplified fragments length polymorphisms (AFLP) and chloroplast DNA have been used to study the species relationships in the genus *Hordeum* as well as revealing genetic diversity in these species (Jaaska 1994, Marillia and Scoles 1996, Nevo *et al.* 1997, Pakniyat *et al.* 1997, Badr *et al.* 2000, Echart-Almeida and Cavalli-Molina 2000, Kochieva *et al.* 2001, El-Rabey *et al.* 2002, Fernández *et al.* 2005, Hou *et al.* 2005, Sabine and Blattner 2006, Castillo *et al.* 2008).

Hordeum species grow in the north, north-west and south-west of Iran and are considered as important forage plants of the country. Parsa (1950), Mobayyen (1981) and Bor (1970) reported the occurrence of 11 *Hordeum* species in Iran; however the species recognition is varied by these authors. Our previous cytogenetic study of the genus contained karyotype analysis of some species (Sheidai and Rashid 2007), while the present study considers chiasma frequency and chromosome pairing in 6 populations of 4 species as well as RAPD analysis of 10 species of Iran.

Materials and methods

Plant materials

Hordeum species studied are 1-*H. violaceum* Boiss., 2-*H. bogdanii* Wilensky, 3-*H. brevisubulatum* Link., from the sect. *Stenostachys* Nevski, 4-*H. marinum* subsp. *marinum* Hudson, 5-*H. glaucum* Steud., 6-*H. leporinum* Link., from the sect. *Hordeastrum* Döll., 7-*H. bulbosum* L., from the sect. *Bulbohordeum* Nevski, 8-*H. spontaneum* C. Koch, 9-*H. distichon* L., and 10-*H. vulgare* L., from the sect. *Crithe* Düll. Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU). The names of localities (populations) have been given in caption of Fig. 1.

Cytological study

Meiotic studies were performed on 6 populations of 4 species of *H. bulbosum*, *H. distichon*, *H. spontaneum* (3 populations) and *H. leporinum*. Young flower buds were collected from 10 randomly selected plants of each species/population and fixed in glacial acetic acid:ethanol (1:3) for 24 h. Flower buds were washed and preserved in 70% ethanol at 4°C until used (Sheidai *et al.* 2008). Cytological preparations used squash technique and 2% aceto-orcein. Fifty to 100 pollen mother cells (PMCs) were analyzed for chiasma frequency and distribution at diakinesis and metaphase, and 500 PMCs were analysed for chromosome segregation during the anaphase I and telophase I.

For grouping of the species a combined data set was produced from meiotic data obtained in this study and karyotype data reported before (Sheidai and Rashid 2007). Since the species studied differed in their chromosome number, relative cytological data were used, for this purpose, mean of ring and rod bivalents per cell, mean of univalents per cell and total chiasmata per bivalent were used (Sheidai *et al.* 2008). Similarly karyotype features not affected by the chromosome number, including size of the longest and shortest chromosomes, total form percentage (TF%) and A1 and A2 karyotype indices of Romero-Zarco (Sheidai and Rashid 2007) were used. Cluster analysis using Unweighted Paired Group with Arithmetic Average (UPGMA) and Neighbor Joining (NJ) methods was performed on relative cytological characteristics.

DNA extraction and RAPD analysis

DNA was extracted from leaves collected randomly from fresh plants or the herbarium specimens by use of NucleoSpin Plant kit (Macherey-Nagel, Germany). The PCR reaction mixture consisted of template DNA, 1×PCR buffer (10 mM Tris-HCl pH 8.8, 250 mM KCl), 200 μM dNTPs, 0.80 μM 10-base random primers and 1 unit of *Taq* DNA polymerase, in a total volume of 25 μl. DNA amplification was performed on a palm cycler GP-001 (Corbet, Australia). Template DNA was initially denatured at 92°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92°C, primer annealing for 1 min at 36°C and primer extension for 2 min at 72°C. A final incubation for 10 min at 72°C was performed. The PCR amplified products were separated by electrophoresis on a 2% agarose gels using 0.5×TBE buffer (44.5 mM Tris/borate, 0.5 mM EDTA, pH=8) or 12% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light or silver stained for added sensitivity. RAPD markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs.

Twenty random primers supplied by Operon technology (Alameda, California) were used. In molecular analysis, only unambiguous and stable PCR fragments that consistently appeared in 2 replications were scored as binary characters and coded accordingly (presence=1, absence=0). Grouping of the species was performed by using different clustering methods based on Dice and Jaccard similarity indices determined among the species studied (Ingrouille 1986, Podani 2000). The fit of dendrograms obtained by different clustering methods was checked by cophenetic correlation (Ingrouille 1986, Podani 2000). NTSYS ver. 2.1 (1998) was used for statistical analyses.

Results and discussion

RAPD analysis

Out of 20 RAPD primers used 11 produced 18 reproducible polymorphic bands (Fig. 1). Specific bands were observed in some of the species or populations for example band B05-1 occurred only in *H. brevisbulatum* and populations of *H. glaucum*, while band R12-1 was present only in *H. bogdanii* and band R12-3 occurred only in the Khoozestan population of *H. marinum*.

Some bands occurred in all the species except 1, for example bands B05-3 and B05-4 was present in all except *H. brevisbulatum*, RAPD band A11-1 was present in all except in *H.*

brevisbulatum and *H. violaceum*, while band A11-4 was absent only in *H. brevisbulatum*, the Evin population of *H. leporinum* and *H. violaceum*.

Two clustering methods performed on RAPD data produced similar results and since the NJ dendrogram showed the highest cophenetic correlation ($r > 0.86$) (Fig. 2). The Dendrogram obtained showed separation of *H. vulgare* and *H. spontaneum* although these species show more affinity compared to the other species studied. Taxonomists variously consider *H. vulgare* ssp. *vulgare* and *H. vulgare* ssp. *spontaneum* as 2 subspecies or as species. *H. spontaneum* has been identified either as an ancestral form or as a feral form of cultivated barley; however many believe that *H. vulgare* is descended from wild barley *H. spontaneum* (Badr *et al.* 2000, Young 2001). A study of RAPD analysis by Kochieva *et al.* (2001) also revealed high similarity between *H. vulgare* and *H. spontaneum* and considered them as 2 subspecies that are well differentiated.

H. spontaneum is an annual plant with diploid and self-pollinating. The genetic diversity of *H. spontaneum* has been identified by isozyme polymorphisms (Liu *et al.* 2002) RFLP markers (Zhang *et al.* 1993), SSR-markers (Matus and Hayes 2000), AFLP markers (Pakniyat *et al.* 1997) and SNP-markers (Kanazin *et al.* 2002). *H. spontaneum* shows more variation than cultivated barley, and many alleles are associated with specific environments and distinct geographic patterns of genetic diversity are maintained in wild barley (*H. spontaneum*) despite migration (Morrell *et al.* 2003). Separation of *H. spontaneum* and *H. vulgare* populations in different clusters obtained in the present study also supports such inter-population variations.

In recent taxonomic treatments of the sea barley (*H. marinum* S.L.), 2 entities have been recognized namely *H. marinum* and *H. geniculatum*, either at the species level or at the sub-specific level (Sahebi *et al.* 2004). Based on gross morphology, the 2 entities differ by the shape of the glumes of the lateral spikelets, being heteromorphous in *H. marinum* and homomorphous in *H. geniculatum*, i.e., the lower glume is wide-winged in the former and without such a wing in the latter. In the present study only *H. marinum* subsp. *marinum* could be collected and included for RAPD analysis.

Three species of *H. marinum* subsp. *marinum*, *H. glaucum* and *H. leporinum* from the sect. *Hordeastrum* are grouped together, *H. marinum* and *H. leporinum* are placed close to each other in a single cluster, while *H. glaucum* joins them with some distance. RFLP of chloroplast DNA also separated *H. glaucum* from *H. leporinum* and *H. marinum* (Baum and Bailey 1989). In other studies, RAPD analysis (De Bustose *et al.* 1998) also showed close affinity of *H. marinum* and *H. leporinum* while AFLP analysis (El-Rabey *et al.* 2002) showed close affinity of *H. glaucum* and *H. leporinum*.

Three species of *H. violaceum*, *H. bogdanii* and *H. brevisbulatum*, from the sect. *Stenostachys* with the H genome are placed in different clusters separated from each other. Therefore RAPD data is not congruent with morphological characters grouping of these species. This result may support Bothmer *et al.* (1995) suggestion that cytogenetic and biochemical data produce different evolutionary hierarchies compared to that of morphological characters in the *Hordeum*. Fernández *et al.* (2005) also agree with Bothmer *et al.* (1995) suggestion; they studied biochemical variation to determine phylogenetic relationships between *Hordeum chilense* and other American species of the *Hordeum* using slab gel electrophoresis techniques and concluded that: 1) Conventional classification based on morphological characters does not totally agree with biochemical data, 2) Sect. *Anisolepis* and *Critesion* seem to be clearly differentiated, 3) The accessions classified as *H. compressum* present biochemical phenotypes quite different from the rest of the species, 4) *H. stenostachys*, *H. muticum* and *H. chilense* constitute a group of variable species with many biochemical similarities and close phylogenetic relationships and 5) The evolutionary pattern of these American species seems to follow a model of reticulate evolution.

Fig. 1. RAPD profile of primers OPA11 (top) and OPR02 (bottom) in *Hordeum* species studied. Abbreviations: NO=No DNA, BE1=Lorestan population of *H. brevisubulatum*, G1, G2=Tehran and Isfahan populations of *H. glaucum*, L1, L2=Tehran and Mazandaran populations of *H. leporinum*, M1, M2=Khoozestan and Gilan populations of *H. marinum* subsp. *marinum*, BU1=Tehran population of *H. bulbosum*, LAD=Molecular ladder, BU2=Lorestan population of *H. bulbosum*, S1, S2=Shiraz and Golestan populations of *H. spontaneum*, BOG=Kerman population of *H. bogdanii*, V1, V2=Isfahan and Mazandaran populations of *H. vulgare*, D1, D2=Tehran and Khoozestan populations of *H. distichon* and VIO=Gilan population of *H. violaceum*.

Cytology

The chromosome number and meiotic details of the *Hordeum* species and populations studied are presented in Table 1 and Fig. 3. The Tehran population of *H. bulbosum* possessed $2n=4x=28$ supporting the earlier reports (Strid and Franzen 1981, Semenov 1986). Bothmer (1979) reported $2n=28$ and $2n=42$ for *H. bulbosum*. Previously Sheidai and Rashid (2007) based on karyotype study reported $2n=2x=14$ for Tehran population of *H. bulbosum*, while the present meiotic study of the same population shows the presence of tetraploid indicating the occurrence of 2 polyploidy level for this species in Iran.

The Darabad population of *H. distichon* possessed $2n=2x=14$ chromosome number, supporting earlier reports (Zhang *et al.* 1990, Sheidai and Rashid 2007). The Evin population of *H. leporinum* possessed $2n=6x=42$ chromosome number. The earlier studies reported diploid and tetraploid chromosome numbers for this species (Hatch 1980, Valdés *et al.* 1999), while recently Sheidai and Rashid (2007) reported hexaploid for this species based on karyotype study of the Shahzand population in Iran which is supported by present meiotic analysis. Three populations of *H. spontaneum* studied showed $2n=2x=14$ supporting earlier reports (Kahler *et al.* 1981, Jahan *et*

Fig. 2. NJ dendrogram of *Hordeum* species based on RAPD data. Species localities as in Fig. 1.

al. 1992, Sheidai and Rashid 2007).

Meiotic analysis of *Hordeum* species and populations studied showed that the chromosomes mainly formed bivalents at metaphase I (Figs. 3A–D). Chromosomes segregation during anaphase was normal in most of the anaphase and telophase cells in all the species studied, except in a few cases of laggard chromosomes formation and chromosome stickiness observed in *H. bulbosum* (Figs. 3E, F). Laggard chromosomes led to formation of micronuclei in this species (Figs. 3G).

The Darake population of *H. bulbosum* and *H. leporinum* showed 0–2 B-chromosomes which were smaller than A-chromosomes and did not pair with them (Fig. 3H, I). The B-chromosomes (Bs) show numerical polymorphism and when present in high number affect negatively the growth and vigor of the plants, while in low number may benefit the plant possessing them. Since few meiocytes having B chromosomes were obtained in this study we could not analyse the effects of Bs on chiasma frequency and distribution which will be done in future detailed study of Bs in *Hordeum* species and populations. To our knowledge this is the first report on the occurrence of B chromosome in *Hordeum* species.

Bao-Rong (1997) studied chromosome pairing and chiasma frequency in parental species and hybrids of *H. bulbosum*, *H. bogdanii*, *H. vulgare*, *H. stenostachys* and *H. murinum* which all formed mainly bivalents in metaphase of meiosis-I with few cases of univalents. The values obtained for chromosome associations and chiasma frequency were in close range to the values of *Hordeum* species studied by us.

Grouping of the *Hordeum* species by different clustering methods based on relative cytological data (Table 1) produced similar results (Fig. 4) and since the UPGMA dendrogram showed the highest cophenetic correlation (0.84) it is discussed here.

Two species of *H. bulbosum* and *H. vulgare* are separated from each other but do not show

Fig. 3. Representative meiotic cells in *Hordeum* species studied. A=Metaphase cell showing $2n=28$ in *H. spontaneum*, B=Metaphase cell showing $2n=28$ in *H. distichon*, C=Metaphase cell showing $2n=42$ in *H. leporinum*, D=Metaphase cell showing $2n=14$ in *H. spontaneum*, E=Meiocyte showing laggard chromosomes in *H. bulbosum*, F=Meiocyte showing chromosome stickiness in *H. bulbosum*, G=Meiocyte showing micronucleus (arrow) in *H. bulbosum*, H=Meiocyte showing one B-chromosome (arrow) in *H. bulbosum* and I=Meiocyte showing 2 B-chromosomes (arrows) in *H. leporinum*. Scale bar=10 μm

close relationship as suggested in RAPD analysis stated before. The species of *H. spontaneum*, *H. distichon* are placed close to each other as in RAPD analysis supporting their taxonomic position in Flora Iranica (Bor 1970), but *H. vulgare* is placed far from them.

Three species of *H. marinum* subsp. *marinum*, *H. glaucum* and *H. leporinum* from the sect. *Hordeastrum* show similarity and are placed close to each other. *H. marinum* and *H. glaucum* show

Table 1. Relative meiotic characters and karyotype features in *Hordeum* species studied

	2n	RBN	RDN	IN	TXN	L	S	TF	A1	A2
<i>H. bogdanii</i>	14	0.93	0.05	0.02	1.95	—	—	—	—	—
<i>H. vulgare</i>	14	0.97	0	0	1.97	31.7	14.55	38.86	0.36	25.96
<i>H. spontaneum</i>	14	0.75	0.25	0	1.75	19.33	12.49	41.97	0.27	16.54
<i>H. bulbosum</i>	28	0.41	0.38	0	1.91	20.5	13.75	41.9	0.26	16.49
<i>H. leporinum</i>	42	0.80	0.18	0	1.8	23.25	11	40.11	0.3	22.97
<i>H. distichon</i>	14	0.73	0.32	0	1.77	22.87	14.49	40.34	0.29	16.75
<i>H. glaucum</i>	14	—	—	—	—	20.25	12	40.2	0.31	16.49
<i>H. marinum</i>	14	—	—	—	—	19.18	10.25	38.25	0.36	20.64

RBN=Ring bivalent per cell, RDN=Rod bivalent per cell, IN=Univalent bivalent per cell, TXN=Total chiasmata per chromosome, L=Size of the longest chromosome (μm), S=Size of the shortest chromosome (μm), TF=Total form percentage, A1=Intra-chromosomal index, A2=Inter-chromosomal index.

Fig. 4. UPGMA clustering of *Hordeum* species based on relative cytological data.

more similarity in contrast with RAPD analysis explained before. The species of *H. marinum* subsp. *marinum* has X genome, while *H. glaucum* and *H. leporinum* contains H genome. In general it seems that cytological data used also show gross affinity of the *Hordeum* species studied which is almost in general agreement with RAPD data obtained.

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