Certification

Molecular Phylogeny of the genus *Hordeum* and origins of *Hordeum* polyploidy species

by

Huan Wang

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Approved:	Dr. Genlou Sun Supervisor Department of Biology
Approved:	Dr. Gavin Kernaghan External Examiner Department of Biology Mount Saint Vincent University
Approved:	Dr. Zhongmin Dong Supervisory Committee Member Department of Biology
Approved:	Dr. Lisa Gannett Supervisory Committee Member Department of Philosophy
Approved:	Dr. Jason Clyburne Program Representative
Approved:	Dr. Jeremy Lundholm Dean of Graduate Studies Representative
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Molecular Phylogeny of the genus *Hordeum* and origins of *Hordeum* polyploid species

By Huan Wang

Date of Submission: March, 2011

Abstract: The phylogeny of the diploid species in the genus *Hordeum* has been studied intensively, however, there has been incongruences between nuclear and chloroplast datasets. In addition, the origins of polyploid species in *Hordeum* remain unclear. The aims of the present study are to: 1) Investigate the phylogeny of *Hordeum* diploid species. 2) Investigate the origins of *Hordeum* polyploids, with combined genetic information from both chloroplast and nuclear datasets. Thirty two *Hordeum* species from eighty accessions were used in this study. In total 214 sequences from three single copy nuclear genes and two chloroplast regions were obtained. Both nuclear and chloroplast phylogenies of *Hordeum* diploids are supported by previous studies, and our study suggests the major incongruence between them could be explained by incomplete lineage sorting. For polyploids, our study confirms *H. brachyantherum* subsp. *californicum* is the parent of *H. brachyantherum* subsp. *brachyantherum* and *H. depressum*. The present study does not favor previous results that *H. roshevitzii* is the possible parent of *H. tetraploidum* and *H. fuegianum*. Instead, this study suggests *H. brevisubulatum* is the possible genome donor to tetraploids *H. brachyantherum* subsp. *brachyantherum, H. tetraploidum, H. fuegianum* and hexaploids *H. lechleri* and *H. procerum*. The present study also suggests the other genome donor of *H. tetraploidum* and *H. fuegianum* might come from *H. patagonicum* species. The diploid *H. pusillum* is further confirmed as the maternal parent of *H. arizonicum* in the present study. Previous suggestion on *H. tetraploidum* as the possible tetraploid genome donor of *H. parodii* and *H. procerum* is supported by our study. In addition, the study also suggests *H. flexuosum* is one possible genome donor of hexaploid *H. parodii*.

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1. Introduction.

Resolving reticulate relationships among individuals, populations and species presents an interesting challenge to evolutionists, and also leads to discoveries of undetected evolutionary processes. Gene trees are the typical tools to infer species evolutionary history, and each gene dataset provides independent phylogenetic information. Unfortunately, different genes sometimes support different trees, thus yielding genetic conflicts in determining the phylogenetic relationship among species of interests. By combining multiple gene datasets and interpreting the incongruences among distinct gene trees, we could clarify historical relationships among species and better classify populations at all levels.

Hordeum has been considered an important model genus for plant phylogenetic study, not only because it includes the economically important crop barley but also because it has a reticulate evolutionary history involving hybridization, polyploidization and introgression. Therefore, a better understanding of the phylogeny of *Hordeum* species will make a great contribution to future plant phylogenetic study. In addition, the investigation of *Hordeum* polyploid origins, based on the genetic information of both nuclear and chloroplast sequences, is also of great significance for future study in other plant populations.

1.1 Phylogenetics

In biology, phylogenetics refers to the study of evolutionary relatedness among different populations at all levels (for example, species, genus, family), through molecular sequencing data and morphological information. Biologists regard evolution as a branching process. They believe populations change over time and may speciate into separate branches, hybridize together or go extinct. The phylogenetic tree is the typical tool to visualize all these evolutionary processes in species history.

In the history of plant evolution, polyploidy is a significant evolutionary force and speciation process. Polyploidy refers to the presence of more than two genomes per cell (Soltis and Soltis, 2000), which is a common phenomenon especially in plants. Polyploidy is detected to have occurred in almost seventy percent of all angiosperms (Masterson, 1994; Wendel, 2000). Many economically important crops including wheat, potato and cotton are polyploids. There are different types of polyploids defined by Stebbins (1950). Allopolyploids are generated by combining two or more distinct genomes while autopolyploids come from the duplication of a single complete genome (Masterson, 1994; Soltis and Soltis 1999, 2000).

Interspecific hybridization and polyploidization have played a central role in the history of plant evolution, and contribute greatly to plant diversification and speciation (Cui *et al.*, 2006). Much attention has been drawn to studying the evolutionary consequences of polyploid species in both genome size and contents,

with the advances in molecular methods over the last two decades (Wendel, 2000; Osborn *et al.*, 2003). Polyploid genome origins and evolution have also been the focus of plant evolutionists (Soltis and Soltis, 1999; Soltis *et al.*, 2003). Increasing evidence has demonstrated the complexity of the dynamic nature of polyploids. Many polyploids are proved to involve multiple origins in space and time (Soltis and Soltis, 1999; Soltis *et al.*, 2003), together with introgression (Mason-Gamer, 2004, 2008; Lihová *et al.*, 2006), while others are believed to have a single origin. Mason-Gamer (2004, 2008) reported that gene introgression could result in unexpected gene copies in the genome, suggesting extensively reticulate relationships in Triticeae species. In addition, polyploidization could also activate transposon elements leading to the increase in genome size, while other mechanisms lead to genome downsizing (Kellogg and Bennetzen, 2004; Leitch and Bennett, 2004).

Incongruence among Distinct Genetic Datasets: The aim of classical molecular phylogenetics is to infer species evolutionary history by reconstructing gene trees based on sequence variation of related species. Modern molecular technology mostly relies on two genetic information pools to investigate the evolutionary relatedness of related plant species or populations—plastid DNA and nuclear markers, each of which has its own merits. However, the attempt to build an accurate species tree often fails, due to incongruences, or even conflicts, between plastid and nuclear genetic phylogenetic information. Such discrepancies can serve as a reflection of biological processes in evolutionary history. There are three major

evolutionary mechanisms potentially resulting in the discordance of different gene phylogenies: incomplete lineage sorting, hidden paralogy, and horizontal gene transfer (Galtier and Daubin, 2008). Incomplete lineage sorting, as perhaps the most studied mechanism, results from retention and stochastic sorting of ancestral polymorphisms, and the difficulties it imposes on interpreting the true species tree have been well described (Pamilo and Nei, 1988; Rosenberg, 2002; Maddison and Knowles, 2006; Meng and Kubatko, 2009). Such phenomena are also present in the genus *Hordeum* (Nishikawa *et al.,* 2002; Petersen and Seberg, 2003; Jakob and Blattner, 2006).

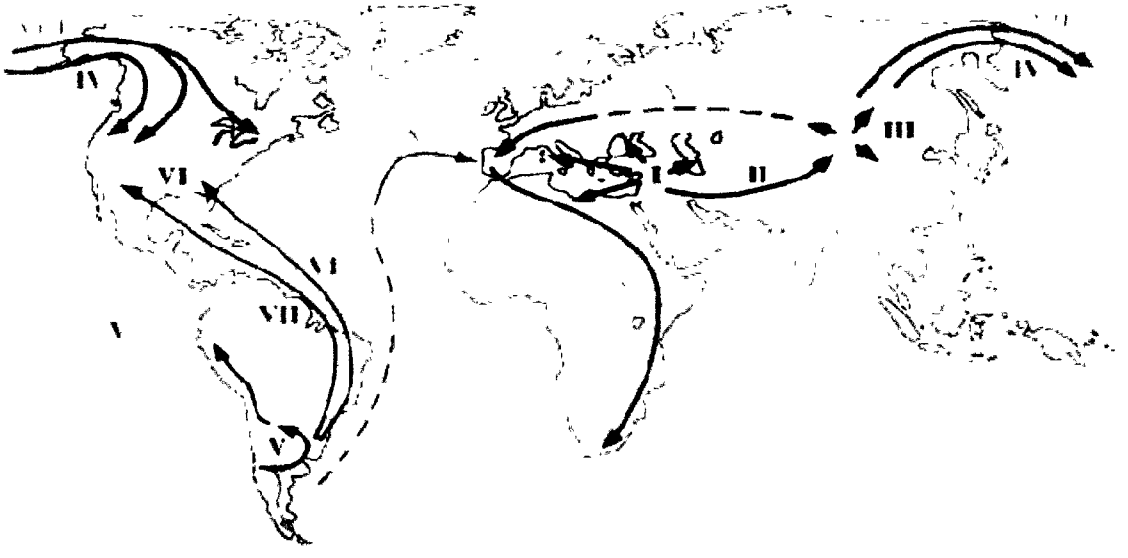
1.2 *Hordeum*

Tribes of the grass family Poaceae have been investigated intensively for the purpose of phylogenetic study because they include a great number of economically important crops and they have proven to have a reticulate evolutionary history. Triticeae, one tribe of Poaceae, comprises the world's most important crops, including barley and wheat, as well as hundreds of related species.

The genus *Hordeum* in Triticeae, which includes 32 species with a basic chromosome number of x=7, is distributed disjunctively in southern South America, South Africa, and the northern hemisphere (Fig. 1) (von Bothmer *et al.*, 1995, Blattner, 2006). Intensive studies have been carried out to investigate the phylogenetic relationship among *Hordeum* species, including morphology, meiotic

Figure 1

Worldwide distribution of *Hordeum* species. The arrows suggest the history of dispersal in *Hordeum* distribution (Blattner, 2006).



chromosome pairing in interspecific hybrids (von Bothmer *et al.*, 1986, 1987, 1988), karyotype and C- banding patterns (Linde-Laursen *et al.,* 1992, 1995), as well as nuclear and chloroplast DNA sequences (Doebley *et al.,* 1992; El-Rabey *et al.*, 2002; Nishikawa *et al.,* 2002; Petersen and Seberg, 2003). Based on karyotype analyses of chromosome types and meiotic chromosome pairing studies of hybrids (von Bothmer *et al.* 1995; Linde-Laursen *et al.*1992), all *Hordeum* species were designated into one of the four basic genome groups, I, H (*Hordeum bulbosum*; *Hordeum vulgare*), X_a (*Hordeum marinum*), and X_u (*Hordeum murinum*) (genome denomination following Blattner, 2009). Isoenzyme analysis (JØrgensen, 1986), restriction site variation in chloroplast DNA (Baum and Bailey, 1991), restriction fragment length polymorphism with repetitive DNA (Svitashev *et al.*, 1994) and DNA sequence data (Petersen and Seberg, 2003; Blattner, 2004) also support the four basic genome groups.

Being the largest genome group, the I genome group includes 14 diploid species, 7 tetraploid species, 4 hexaploid species, and 2 species existing at three ploidy levels (2x, 4x, 6x). Although distributed widely from central Asia to the American continent, I genome species share many morphological characteristics. There have been numerous studies on the phylogenetic relationships of the *Hordeum* diploid species, which are believed to have originated from South-west Asia and spread into Europe and Central Asia (Blattner, 2006). Accumulating evidence supports the monophyletic clade of western Asian and Mediterranean species of the H and X_u genome groups, as well as another monophyletic clade of Eurasian *H. marinum* in

X_a genome group and I genome taxa (Komatsuda *et al.*, 1999; Petersen and Seberg, 2003; Sun *et al.*, 2009). Chloroplast DNA sequence data divided the I genome group *Hordeum* species into "New World" and "Old World" groups (Doebley *et al.*, 1992; Nishikawa *et al.*, 2002).

Although the monopoly of the genus is well supported by molecular phylogenetic studies (Petersen and Seberg, 1997; Seberg and Frederiksen, 2001; Blattner, 2004), the intrageneric phylogeny is still a matter of controversy. The detailed phylogenetic relationships among *Hordeum* species have not fully been understood, largely due to the incongruence between chloroplast and nuclear phylogenies. While nuclear data sets of *Hordeum* species often arrive at similar conclusions (Petersen and Seberg, 2003; Blattner, 2004, 2006; Kakeda, 2009; Sun *et al.*, 2009), studies of chloroplast DNA generally incur conflicts (Doebley *et al.*, 1992; Nishikawa *et al.*, 2002; Petersen and Seberg, 2003; Jakob and Blattner, 2006). Incomplete lineage sorting has been considered as the main cause (Petersen and Seberg, 2003; Jakob and Blattner, 2006).

As for the origins of the polyploids in *Hordeum,* more research is in great demand. Based on Fluorescent *In Situ* Hybridization (FISH) and rDNA-RFLP patterns, Taketa *et al.* (2001, 2005) suggested *H. roshevitzii* and *H. brachyantherum* subsp. *californicum* as the common ancestors of tetrapolyploid species *H. jubatum*, *H. fuegianum*, *H. tetraploidum* and *H. brachyantherum* subsp. *brachyantherum*, and also indicated a close relationship of tetraploid species *H. jubatum* to I genome hexaploid species. Blattner (2006) also supported *H. roshevitzii* and *H. brachyantherum* subsp. *californicum* as the ancestors to *H. jubatum*. However, the

origins of other polyploid species remain unclear. Therefore, further studies on the origins of *Hordeum* polyploid species are necessary.

1.3 Molecular Markers

1.3.1 Chloroplast DNA

Chloroplast DNA (cpDNA) used to be the most widely used genetic source to study plant phylogeny. The main advantage of cpDNA lies in its relatively simple inheritance. In addition, the high copies of cpDNA genes make it very easy to conduct in restriction site analysis as well as gene amplification. However, cpDNA follows maternal inheritance, and such uniparental inheritance allows it to be only able to reveal half of the parentage in hybrid or polyploid plants (Olmstead and Pamer, 1994; Soltis and Soltis, 1998)

1.3.2 Single Copy Nuclear DNA

Single copy nuclear DNA nowadays has been regarded as the ideal candidate for phylogenetic study, especially in identifying donors of hybrids or polyploids (Sang, 2002). This is because: 1) nuclear genes evolve faster than organellar genomes (Wolfe *et al.*, 1987; Gaut 1998). 2) Nuclear genes are likely to have experienced more independent evolution events, for example hybridization and introgression, and therefore they possess higher detected variation. Combining several single copy nuclear datasets thus could greatly enhance the accuracy of phylogenetic study. 3) Compared to nuclear ribosomal DNA (rDNA), single

copy nuclear DNA is much less susceptible to concerted evolution (Small *et al.*, 2004). This characteristic is especially important in investigating polyploid origins, because polyploids are expected to possess multiple gene copies. 4) nuclear gene follows biparental inheritance and provides information on both parents.

1.4 The Aims of This Study

To better understand the evolutionary history of *Hordeum* species by adding both chloroplast and nuclear data sets, two chloroplast gene loci, trnD-trnT intergenic spacer and rps16 gene, as well as three nuclear markers, including thioreoxin-like gene (HTL), disrupted meiotic cDNA (DMC1), and the gene encoding the second subunit of RNA polymerase II (RPB2), were used to explore the phylogeny of *Hordeum* species and investigate the origins of *Hordeum* polyploids in the present study. The aims of the present study are to: 1) Investigate the phylogeny of *Hordeum* diploid species with both chloroplast and nuclear data sets. 2) Investigate the origins of *Hordeum* polyploids with combined genetic information from both chloroplast and nuclear datasets. In addition, the information generated in the present study could also help us better understand the historical process of incomplete lineage sorting and how it results in incongruence between nuclear and chloroplast phylogenies.

2. Materials and Methods

2.1 Materials

Thirty two *Hordeum* species from eighty accessions were used in this study. In total 214 sequences from three single copy nuclear genes and two chloroplast regions were obtained, including a few sequences downloaded directly from GenBank. Seeds were ordered from NordGen Institution in Sweden. Seeds were germinated on absorbent paper in Petri dishes, and then transplanted to a sand-peat mixture in a greenhouse. The species name, accession no., origins, genome and ploidy are listed in Table 1.

Table 1

Taxa used in this study. The species name, accession no., origins, genome and ploidy are listed here. Sequences from the species with * are directly downloaded from GenBank.

Species	Accession No.	Origin	Genome	Ploidy
Hordeum arizonicum	H 2144	Mexico		6x
Hordeum brachyantherum subsp. *Brachyantherum*	H 2318	USA		4x
Hordeum brachyantherum subsp. *Brachyantherum*	H 2348	USA		4x
Hordeum brachyantherum subsp. *californicum* *	H 1942	USA	I	2x
Hordeum brachyantherum subsp. *californicum*	H 1954	USA	I	2X
Hordeum brachyantherum subsp. *californicum*	H 3317	USA	I	2x
Hordeum brevisubulatum *	H 304	Turkey	I	2x
Hordeum brevisubulatum *	H 316	Iran	I	2x
Hordeum brevisubulatum	H 10056	Russia	I	2x
Hordeum brevisubulatum subsp. *violaceum* *	H 315	Iran	I	2x
Hordeum bogdanii *	H 4014	Pakistan	I	2x
Hordeum bogdanii	H 7476	China	I	2x
Hordeum bulbosum *	H 3878	Italy	H	2x
Hordeum bulbosum *	710-17	Morocco	H	2x
Hordeum capense	H 334	South Africa		4x
Hordeum chilense	H 1819	Chile	I	2x
Hordeum chilense *	Camb.line 1	Unkown	I	2x
Hordeum comosum *	H 1181	Argentina	I	2x
Hordeum comosum	H 10608	Argentina	I	2x
Hordeum cordobense	H 1702	Argentina	I	2x
Hordeum cordobense *	H 6429	Argentina	I	2x
Hordeum depressum	H 2008	USA		4x
Hordeum depressum	H 2089	USA		4x
Hordeum erectifolium	H 1150	Argentina	I	2x
Hordeum euclaston	H 1103	Argentina	I	2x
Hordeum euclaston	H 1263	Argentina	I	2x
Hordeum flexuosum	H 1112	Argentina	I	2x
Hordeum flexuosum *	H 1133	Argentina	I	2x
Hordeum fuegianum	H 1376	Chile		4x
Hordeum fuegianum	H 1418	USA		4x
Hordeum fuegianum	H 6168	Argentina		4x
Hordeum intercedens *	H 1940	USA	I	2x
Hordeum intercedens	H 2310	USA	I	2x
Hordeum jubatum	H 1162	Argentina		4x
Hordeum jubatum	H 2013	Mexico		4x

Hordeum lechleri	H 1451	Chile		6x
Hordeum lechleri	H 6344	Argentina		6x
Hordeum marinum subsp. *gussoneanum*	H 160	Portugal	Xa	2x
Hordeum marinum subsp. *gussoneanum* *	H 299	Bulgaria	Xa	2x
Hordeum marinum subsp. *gussoneanum* *	H 539	Spain	Xa	2x
Hordeum marinum subsp. *marinum* *	H 515	Spain	Xa	2x
Hordeum marinum subsp. *marinum* *	H 546	Spain	Xa	2x
Hordeum marinum subsp. *marinum*	H 559	Spain	Xa	2x
Hordeum murinum subsp. *glaucum*	H 52	Jordan	Xu	2x
Hordeum murinum subsp. *glaucum* *	H 801	Iran	Xu	2x
Hordeum murinum subsp. *glaucum* *	H 10289	Tajikistan	Xu	2x
Hordeum muticum *	H 958	Bolivia	I	2x
Hordeum muticum	H 6470	Argentina	I	2x
Hordeum parodii	H 1146	Argentina		6x
Hordeum parodii	H 1458	Argentina		6x
Hordeum patagonicum subsp. *magellanicum*	H 1363	Argentina	I	2x
Hordeum patagonicum subsp. *magellanicum*	H 1368	Chile	I	2x
Hordeum patagonicum subsp. *magellanicum* *	H 6209	Argentina	I	2x
Hordeum patagonicum subsp. *mustersii*	H 1358	Argentina	I	2x
Hordeum patagonicum subsp. *patagonicum* *	H 1319	Argentina	I	2x
Hordeum patagonicum subsp. *patagonicum*	H 1520	Argentina	I	2x
Hordeum patagonicum subsp. *santacrucense*	H 1462	Argentina	I	2x
Hordeum patagonicum subsp. *santacrucense* *	H 1493	Argentina	I	2x
Hordeum patagonicum subsp. *setifolium*	H 1352	Argentina	I	2x
Hordeum patagonicum subsp. *setifolium* *	H 1357	Argentina	I	2x
Hordeum procerum	H 1156	Argentina		6x
Hordeum procerum	H 1166	Argentina		6x
Hordeum pubiflorum *	H 1296	Argentina	I	2x
Hordeum pubiflorum	H 1379	Chile	I	2x
Hordeum pusillum	H 1901	USA	I	2x
Hordeum pusillum *	H 2038	USA	I	2x
Hordeum roshevitzii *	H 7202	China	I	2x
Hordeum roshevitzii *	H 9152	China	I	2x
Hordeum roshevitzii	H 10070	Russia	I	2x
*Hordeum secalinum**	H 231	Sweden		4x
Hordeum stenostachys *	H 1783	Argentina	I	2x
Hordeum stenostachys	H 6439	Argentina	I	2x
Hordeum tetraploidum	H 6198	Argentina		4x
Hordeum tetraploidum	H 6364	Argentina		4x
Hordeum vulgare	H 7405	China	H	2x
Hordeum vulgare subsp. *spontaneum* *	H 3139	Cyprus	H	2x
Hordeum vulgare subsp. *Spontaneum*	H 3173	China	H	2x

Hordeum vulgare subsp. *spontaneum* *	OUH620	Mexico	H	2x
Hordeum vulgare subsp. *vulgare* *	H 5867	Cultivated	H	2x
Hordeum vulgare subsp. *vulgare* *	Bonus	Sweden	H	2x
Bromus arvensis *	C 618			
Bromus catharticus *	CN32048			
Bromus sterilis *	OSA420	Denmark		
Pseudoroegneria spicata *	PI232140	USA	St	
Psathyrostachys juncea *	H 10108	Russia	Ns	

2.2 Methods

2.2.1. DNA Isolation

After being frozen in liquid nitrogen, young leaves were ground with a mortar and pestle. The powder was distributed to 2-mL tubes. Seven hundred and fifty microlitres of lysis solution (50mM Tris-HCl, pH7.6; 100mM NaCl; 50mM EDTA; 0.5% SDS) were added to each tube with 10mM β-mercaptoethanol. The mixture was incubated at room temperature for 10-15 min. Four hundred and fifty microlitres of phenol-tris-chloroform (pH=7.5) were added; the different phases were then mixed gently and then separated by centrifugation at 13000 rpm for 5 min. The upper phase was transferred with a cut-off 5-mL tip into a new tube. Four hundred and fifty microlitres of chloroform-isoamyl alcohol (24:1) were added. Tubes were inverted and centrifuged at max speed (15000 rpm) for 5 min. The upper phase was transferred to a new 1.5mL tube. The nucleic acids in the aqueous phase were precipitated by adding 0.6 volumes of cold isopropanol. The tube was incubated for 20 min at -20 °C. The pellet was collected by centrifugation at 13000 rpm for 10 min and then washed with 70% cold ethanol and dried. The dry pellet was dissolved in 400μL of TE (10 mM Tris, pH 8.0; 1mM EDTA, pH 8.0) solution and stored at room temperature for at least 20 hrs before further use. 50μg/mL RNase was added. The tube was then incubated at 37 °C for 30 min. In order to purify the raw genomic DNA that was extracted, a purifying procedure was performed. In this process, steps from above were repeated, from adding Phenol-Tris-Chloroform to resuspension in TE buffer (100 μL TE buffer as final volume). The purity and concentration of the DNA was assessed spectrophotometrically by calculating the A260/280 ratio to determine

protein impurities. The DNA yield was calculated from the A260 for clean DNA samples (A260/A280 between 1.8 and 2.0) (Sun *et al.*, 1997).

2.2.2. DNA Amplification

DNA amplification and sequencing: The desired sequences were amplified by polymerase chain reaction (PCR) with the primer pair of trnD/trnT (Saski *et al.*, 2007), rps16F/rps16R (5'-GTGGTAGAAAGCAACGTGCGACTT-3'/5'-TCGGGATCGAACATCAATTGCAAC-3') (Popp and Oxelman, 2007), trxF/trxR (5'-CGCRRAATATTCCACKTCCC-3'/5'-YTGGTCCCAGTCCTCTTTGG-3') (Kakeda *et al.*, 2000, 2008), P6F /P6FR (5'-TGGGGAATGATGTGTCCTGC-3'/5'-CGAACCACACCAACTTCAGTGT-3') (Denton *et al.* 1998; Sun *et al.*, 2007, 2008, 2009), and TDMC 1e10/TDMC 1e15R (5'-TGCCAATTGCTGAGAGATTTG-3'/ 5'-AGCCACCTGTTGTAATCTGG-3') (Petersen and Seberg, 2000), following the protocols in table 2. Successful nuclear gene PCR products from diploids and all the successful chloroplast gene PCR products from diploids and polyploids were sequenced directly. Sequencing was done commercially by the MACROGEN (Seoul, Korea). To enhance the sequence results, both forward and reverse strands were sequenced independently.

Table 2

The polymerase chain reaction (PCR) protocols of the five primer pairs are listed here.

Primers	Initial	Exponential amplification				Final
	Denaturation	Denaturation	Annealing	Elongation	cycles	Elongation
trnD/trnT	95 °C for 4 min	95 °C for 1 min	60 °C for 1 min	72 °C for 1 min	35-40	72 °C for 10 min
rps16F/rps16R	95 °C for 3 min	95 °C for 40 sec	63 °C for 1 min	72 °C for 1 min	35-40	72 °C for 10 min
trxF/trxR	95 °C for 5 min	95 °C for 1 min	57 °C for 1.5 min	72 °C for 1 min	35-40	72 °C for 10 min
P6F /P6FR	95 °C for 5 min	95 °C for 1 min	52 °C for 2 min	72 °C for 2 min	35-40	72 °C for 10 min
TDMC 1e10/TDMC 1e15R	95 °C for 5 min	95 °C for 1 min	55 °C for 2 min	72 °C for 2 min	35-40	72 °C for 10 min

2.2.3. Cloning

The nuclear gene PCR products from all the *Hordeum* polyploid species were cloned into the TOPO-TA kit from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Ten clones from each species were randomly selected for screening. Each of those ten clones was transferred to 100 μL of LB broth medium with 0.1 $mg \cdot mL^{-1}$ antibiotics and then incubated at 37 °C for 1 hour before using 2 μL for PCR to check for the presence of a successful insert. For those clone solutions that were confirmed to have the insert, 50 μL of the solution were transferred to a 5 ml LB broth test tube (with 0.1 $mg \cdot mL^{-1}$ antibiotics) and incubated at 37 °C overnight. Plasmid DNA extraction was performed using the Promega Wizard Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI), following the manufacturer's instructions. Plasmid DNA was sequenced commercially by the MACROGEN (Seoul, Korea). To enhance the sequence results, both forward and reverse strands were sequenced independently.

2.2.4. Data Analysis

Automated sequence outputs were inspected visually using chromatographs. Multiple sequence alignments were made using ClustalX with default parameters (Thompson *et al.* 1997). Phylogenetic analysis using the maximum-parsimony (MP) method was performed with the computer program PAUP (Swofford 2003). All characters were treated equally and specified as unweighted and unordered. Indels were excluded for phylogenetic analysis. Most-parsimonious trees were obtained by performing a heuristic search using the Tree Bisection-Reconnection (TBR) option with MulTrees

on, and ten replications of random addition sequences with the stepwise addition option. Overall character congruence was estimated by the consistency index (CI), and the retention index (RI). In order to infer the robustness of clades, bootstrap values with 1000 replications (Felsenstein 1985) were calculated by performing a heuristic search using the TBR option with MulTrees on. In addition, maximum-likelihood (ML) method was also performed using PHYML3.0 (Guindon, 2010). Eight substitution models, including JC69, K80, F81, F84, HKY85, TN93, GTR and custom for nucleotides, were tested for both chloroplast and nuclear data sets and eventually we adopted the model with the highest log-likelihood value – GTR, in the present study. The approximate likelihood ratio test (ALR) value was alternative measure of bootstrap value in ML phylogeny.

3. Results

3.1 *Hordeum* Diploids

3.1.1 chloroplast DNA:

trnD-trnT: Twenty trnD-trnT sequences were aligned. Two obviously sequence types were observed, with distinctive sections of approximate 210 base pairs (Fig. 2). Of a total of 926 characters included in the final analysis, 686 characters were constant, 76 characters were parsimony-uninformative, and 164 characters were parsimony-informative. Phylogenetic analysis based on trnD-trnT region sequences was carried out using the MP and ML methods. A strict consensus tree from the 379 most parsimonious trees is shown in Fig. 3, with consistency index=0.747, retention index=0.886. Both the MP and ML analyses resulted in highly similar tree topologies consisting of two major clades. One clade consists of mainly North and South American species except Eurasian species *H. marinum* subsp. *gussoneanum*, with a bootstrap support of 55%. The other clade consists of mainly Asian or Eurasian species except South American species *H. flexuosum*, with a bootstrap value of 100% and ALR of 1.00. Furthermore, *H. cordobense, H. marrinum* subsp. *gussoneanum, H. brachyantherum* subsp. *californicum* and *H. patagonicum* subsp. *setifolium* were grouped together with a bootstrap value of 65% and ALR of 0.94.

Figure 2

Partial sequence alignment of trnD-trnT from *Hordeum* diploid species were displayed here. Two obviously sequence types were observed, with distinctive sections of approximate 210 base pairs. The complete list of type 1 *Hordeum* species includes twelve species, which are displayed in one clade from Fig. 2 with 55% bootstrap support. They are all distributed in the Americas. The complete list of type 2 *Hordeum* species includes eight species, which are displayed in the other clade from Fig. 2 with 100% bootstrap support. They are all distributed in Eurasia.

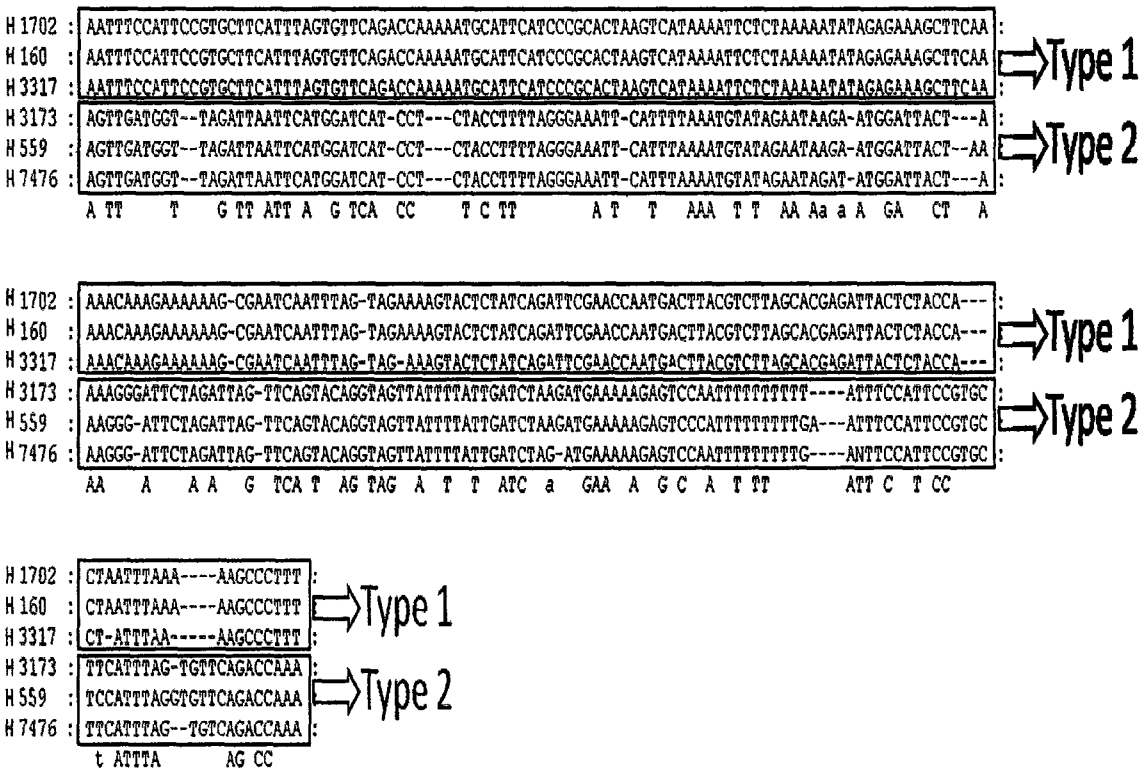
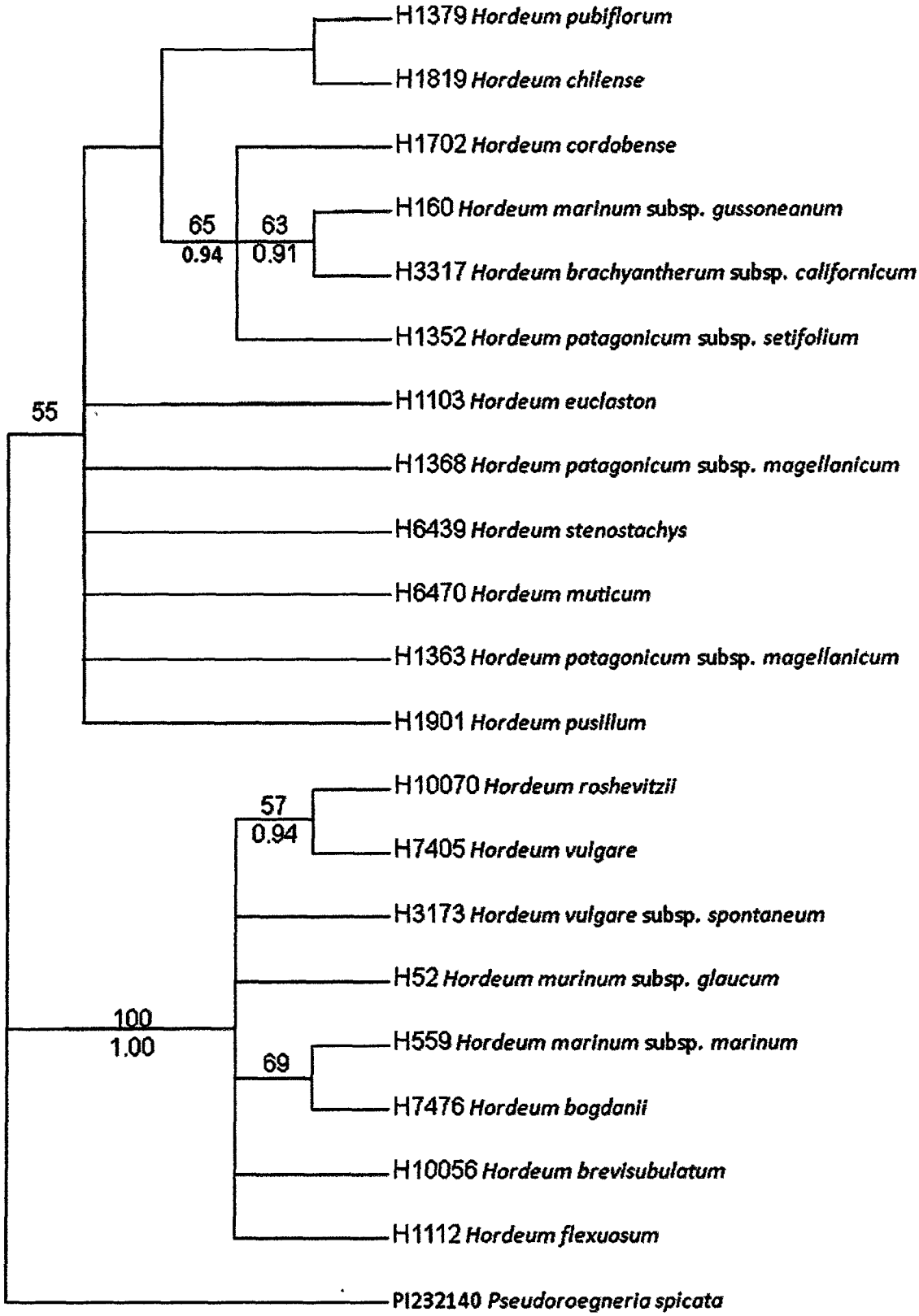

H 1702 : AATTTCCATTCCGTGCTTCATTTAGTGTTCAGACCAAAAATGCATTCATCCCGCACTAAGTCATAAAATTCTCTAAAAATATAGAGAAAGCTTCAA :
H 160 : AATTTCCATTCCGTGCTTCATTTAGTGTTCAGACCAAAAATGCATTCATCCCGCACTAAGTCATAAAATTCTCTAAAAATATAGAGAAAGCTTCAA :
H 3317 : AATTTCCATTCCGTGCTTCATTTAGTGTTCAGACCAAAAATGCATTCATCCCGCACTAAGTCATAAAATTCTCTAAAAATATAGAGAAAGCTTCAA :
H 3173 : AGTTGATGGT--TAGATTAATTCATGGATCAT-CCT---CTACCTTTTAGGGAAATT-CATTTTAAATGTATAGAATAAGA-ATGGATTACT---A :
H 559 : AGTTGATGGT--TAGATTAATTCATGGATCAT-CCT---CTACCTTTTAGGGAAATT-CATTTAAAATGTATAGAATAAGA-ATGGATTACT--AA :
H 7476 : AGTTGATGGT--TAGATTAATTCATGGATCAT-CCT---CTACCTTTTAGGGAAATT-CATTTAAAATGTATAGAATAGAT-ATGGATTACT---A :
A TT T G TT ATT A G TCA CC T C TT A T T AAA T T AA Aa a A GA CT A
Type 1
Type 2
H 1702 : AAACAAAGAAAAAAG-CGAATCAATTTAG-TAGAAAAGTACTCTATCAGATTCGAACCAATGACTTACGTCTTAGCACGAGATTACTCTACCA--- :
H 160 : AAACAAAGAAAAAAG-CGAATCAATTTAG-TAGAAAAGTACTCTATCAGATTCGAACCAATGACTTACGTCTTAGCACGAGATTACTCTACCA--- :
H 3317 : AAACAAAGAAAAAAG-CGAATCAATTTAG-TAG-AAAGTACTCTATCAGATTCGAACCAATGACTTACGTCTTAGCACGAGATTACTCTACCA--- :
H 3173 : AAAGGGATTCTAGATTAG-TTCAGTACAGGTAGTTATTTTATTGATCTAAGATGAAAAAGAGTCCAATTTTTTTTTT----ATTTCCATTCCGTGC :
H 559 : AAGGG-ATTCTAGATTAG-TTCAGTACAGGTAGTTATTTTATTGATCTAAGATGAAAAAGAGTCCCATTTTTTTTTGA---ATTTCCATTCCGTGC :
H 7476 : AAGGG-ATTCTAGATTAG-TTCAGTACAGGTAGTTATTTTATTGATCTAG-ATGAAAAAGAGTCCAATTTTTTTTTG----ANTTCCATTCCGTGC :
AA A A A G TCA T AG TAG A T T ATC a GAA A G C A T TT ATT C T CC
Type 1
Type 2
H 1702 : CTAATTTAAA----AAGCCCTTT :
H 160 : CTAATTTAAA----AAGCCCTTT :
H 3317 : CT-ATTTAA-----AAGCCCTTT :
H 3173 : TTCATTTAG-TGTTCAGACCAAA :
H 559 : TCCATTTAGGTGTTCAGACCAAA :
H 7476 : TTCATTTAG--TGTCAGACCAAA :
t ATTTA AG CC
Type 1
Type 2

Figure 3

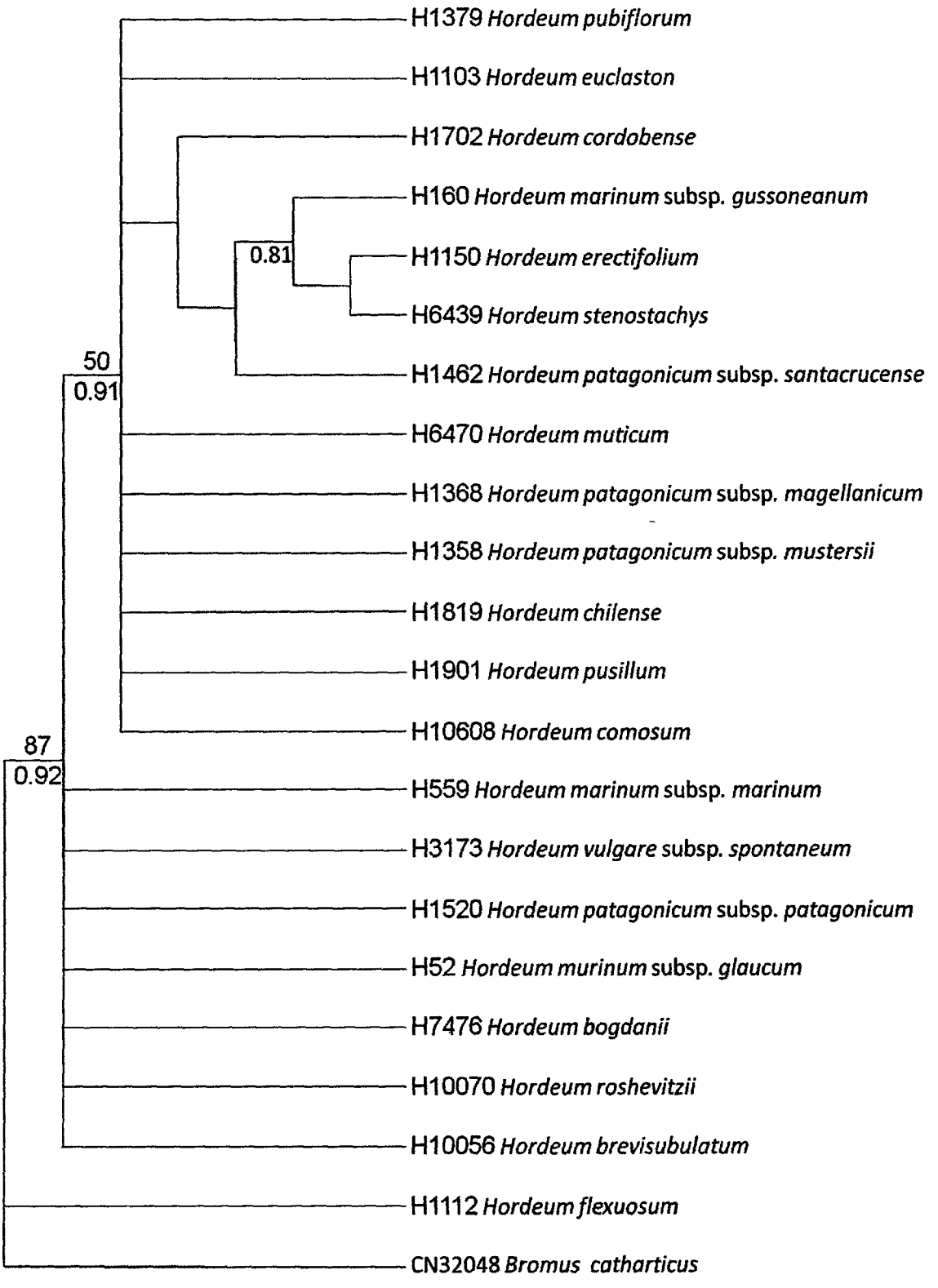
A strict consensus tree in the phylogenetic analysis of trnD-trnT dataset from 379 most parsimonious trees is shown, with consistency index=0.747, retention index=0.886. In total twenty *Hordeum* diploids were included here. Both the MP and ML analyses resulted in highly similar tree topologies consisting of two major clades. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. One clade consists of mainly North and South American species except Eurasian species *H. marinum* subsp. *gussoneanum*, with bootstrap support of 55%. The other clade consists of mainly Asian or Eurasian species except South American species *H. flexuosum*, with bootstrap value of 100% and ALR 1.00. Furthermore, *H. cordobense, H. marrinum* subsp. *gussoneanum, H. brachyantherum* subsp. *californicum* and *H. patagonicum* subsp. *setifolium* were grouped together with bootstrap value of 65% and ALR of 0.94.


H1379 Hordeum pubiflorum
H1819 Hordeum chilense
H1702 Hordeum cordobense
H160 Hordeum marinum subsp. gussoneanum
H3317 Hordeum brachyantherum subsp. californicum
H1352 Hordeum patagonicum subsp. setifolium
H1103 Hordeum euclaston
H1368 Hordeum patagonicum subsp. magellanicum
H6439 Hordeum stenostachys
H6470 Hordeum muticum
H1363 Hordeum patagonicum subsp. magellanicum
H1901 Hordeum pusillum
H10070 Hordeum roshevitzii
H7405 Hordeum vulgare
H3173 Hordeum vulgare subsp. spontaneum
H52 Hordeum murinum subsp. glaucum
H559 Hordeum marinum subsp. marinum
H7476 Hordeum bogdanii
H10056 Hordeum brevisubulatum
H1112 Hordeum flexuosum
PI232140 Pseudoroegneria spicata
65
0.94
63
0.91
55
57
0.94
100
1.00
69

rps16: Sequence comparisons among twenty-one *Hordeum* showed a low level of variation detected in the rps16 region. Of a total of 782 characters analyzed, 732 characters were constant, 42 characters were parsimony-uninformative, and only 9 characters were parsimony-informative. Maximum parsimonious analysis generated 58 trees. A strict consensus tree is shown in Fig. 4, with consistency index=0.948, retention index=0.812. The tree topologies from both MP and ML analyses showed great congruency with each other, and are similar to the results of trnD-trnT analyses. Most North and South American species and one Eurasian species *H. marinum* subsp. *gussoneanum* form the same group, with a bootstrap value of 50% and ALR of 0.91. All the remaining Eurasian and Asian species are sister species to the monophyletic group. Twenty *Hordeum* species formed a group with a bootstrap value of 87% and ALR of 0.92, while the South American species *H. flexuosum* became the sister species to all the other species.

Figure 4

A strict consensus tree in the phylogenetic analysis of rps16 dataset from 58 most parsimonious trees is shown, with consistency index=0.948, retention index=0.812. Twenty-one sequences are from *Hordeum* diploids and the remaining one was used as the outgroup sequence. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. Most North and South American species and one Eurasian species *H. marinum* subsp. *gussoneanum* form a monophyletic group, with a bootstrap value of 50% and ALR 0.91. All the remaining Eurasian and Asian species are sister species to the monophyletic group. Twenty Hordeum species formed a group with a bootstrap value of 87% and ALR 0.92, while the South American species *H. flexuosum* is the sister species to all the other species.

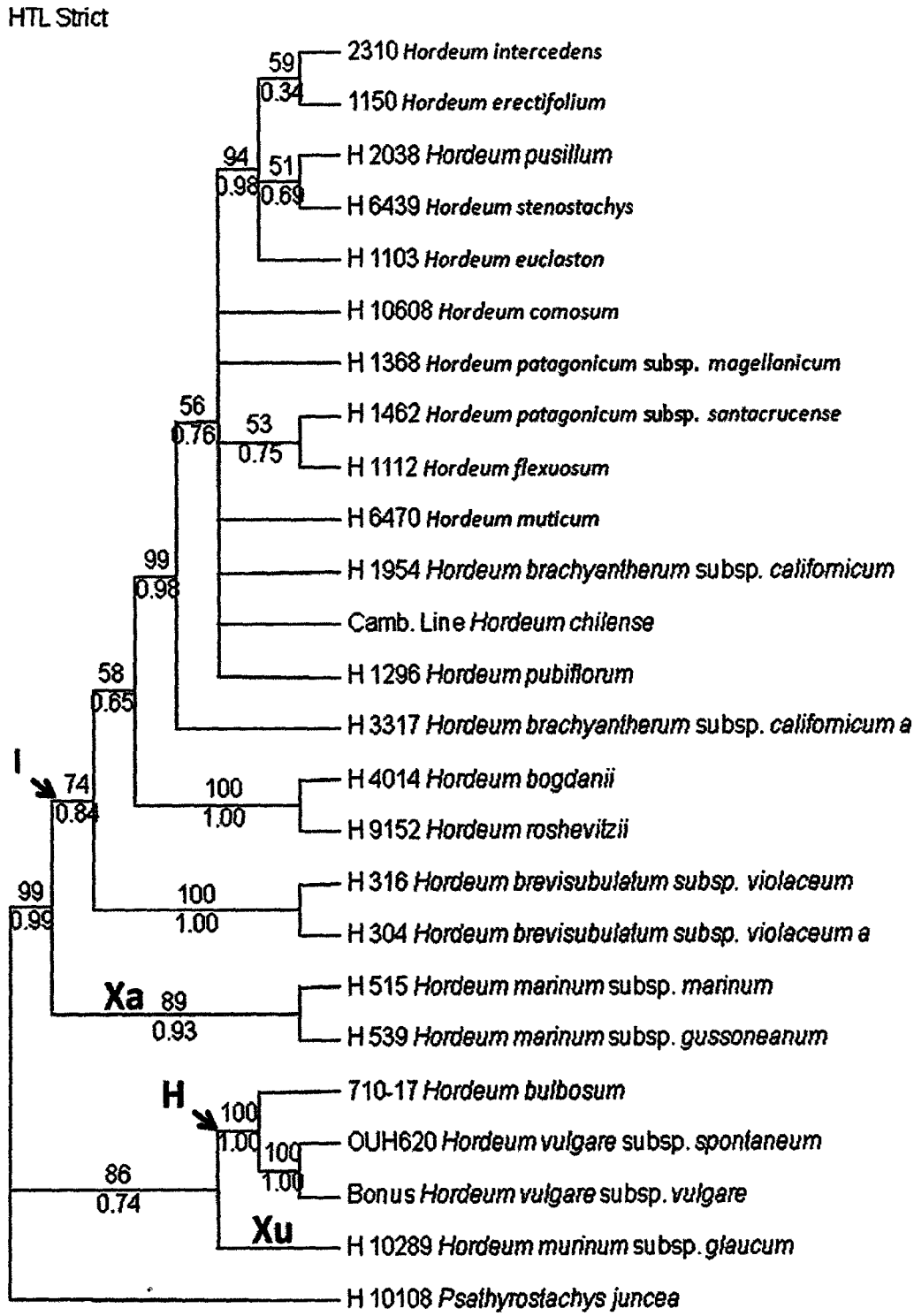

H1379 Hordeum pubiflorum
H1103 Hordeum euclaston
H1702 Hordeum cordobense
H160 Hordeum marinum subsp. gussoneanum
0.81
H1150 Hordeum erectifolium
H6439 Hordeum stenostachys
50
0.91
H1462 Hordeum patagonicum subsp. santacrucense
H6470 Hordeum muticum
H1368 Hordeum patagonicum subsp. magellanicum
H1358 Hordeum patagonicum subsp. mustersii
H1819 Hordeum chilense
H1901 Hordeum pusillum
H10608 Hordeum comosum
87
0.92
H559 Hordeum marinum subsp. marinum
H3173 Hordeum vulgare subsp. spontaneum
H1520 Hordeum patagonicum subsp. patagonicum
H52 Hordeum murinum subsp. glaucum
H7476 Hordeum bogdanii
H10070 Hordeum roshevitzii
H10056 Hordeum brevisubulatum
H1112 Hordeum flexuosum
CN32048 Bromus catharticus

3.1.2. Nuclear Gene:

HTL: Nine sequences were obtained for the HTL, and analyzed together with fifteen HTL sequences downloaded from GenBank. Of a total of 979 characters analyzed, 704 characters were constant, 149 characters were parsimony-uninformative, and 126 characters were parsimony-informative. MP and ML analyses based on the HTL sequences were carried out using *Psathyrostachy juncea* as the outgroup. The results of both MP and ML analyses showed high similarity in tree topologies. A strict consensus tree from the 349 most parsimonious trees is shown in Fig. 5, with consistency index of 0.885, retention index of 0.880. All *Hordeum* diploids were divided into two clades: the Xu and H genome clade (bootstrap value of 86% and ALR of 0.74) and the Xa and I genome clade (bootstrap value of 99% and ALR of 0.99). Within the Xu and H clade, H genome species (*H. bulbosum, H. vulgare* subsp. *spontaneum,* and *H. vulgare* subsp. *vulgare*) formed a group (bootstrap value of 100% and ALR of 1.00) well separated from the Xu (*H. murinum*) species. A clear separation between Xa (*H. marinum* subsp. *marinum* and *H. marinum* subsp. *gussoneanum*, with bootstrap value of 89% and ALR of 0.93) and I genome species (all the remaining species, with bootstrap value of 74% and ALR of 0.84) was also observed. Furthermore, five American species *H. intercedens, H. erectifilium, H. pusillum, H. stenostachys* and *H. euclaston* were grouped together with a bootstrap value of 94% and ALR of 0.98.

Figure 5

A strict consensus tree in the phylogenetic analysis of HTL dataset from 349 most parsimonious trees is shown, with consistency index=0.885, retention index=0.880. Twenty-four sequences from *Hordeum* diploids were included here, of which fifteen sequences were obtained from GenBank. The sequence of species *Psathyrostachy juncea* was used as the outgroup. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis.


HTL Strict
2310 Hordeum intercedens
1150 Hordeum erectifolium
H 2038 Hordeum pusillum
H 6439 Hordeum stenostachys
H 1103 Hordeum euclaston
H 10608 Hordeum comosum
H 1368 Hordeum patagonicum subsp. magellanicum
H 1462 Hordeum patagonicum subsp. santacrucense
H 1112 Hordeum flexuosum
H 6470 Hordeum muticum
H 1954 Hordeum brachyantherum subsp. californicum
Camb. Line Hordeum chilense
H 1296 Hordeum pubiflorum
H 3317 Hordeum brachyantherum subsp. californicum a
H 4014 Hordeum bogdanii
H 9152 Hordeum roshevitzii
H 316 Hordeum brevisubulatum subsp. violaceum
H 304 Hordeum brevisubulatum subsp. violaceum a
H 515 Hordeum marinum subsp. marinum
H 539 Hordeum marinum subsp. gussoneanum
710-17 Hordeum bulbosum
OUH620 Hordeum vulgare subsp. spontaneum
Bonus Hordeum vulgare subsp. vulgare
H 10289 Hordeum murinum subsp. glaucum
H 10108 Psathyrostachys juncea
I
Xa
H
Xu
59
0.34
94
0.98
51
0.69
56
0.76
53
0.75
99
0.98
58
0.65
74
0.84
100
1.00
99
0.99
100
1.00
89
0.93
100
1.00
100
1.00
86
0.74

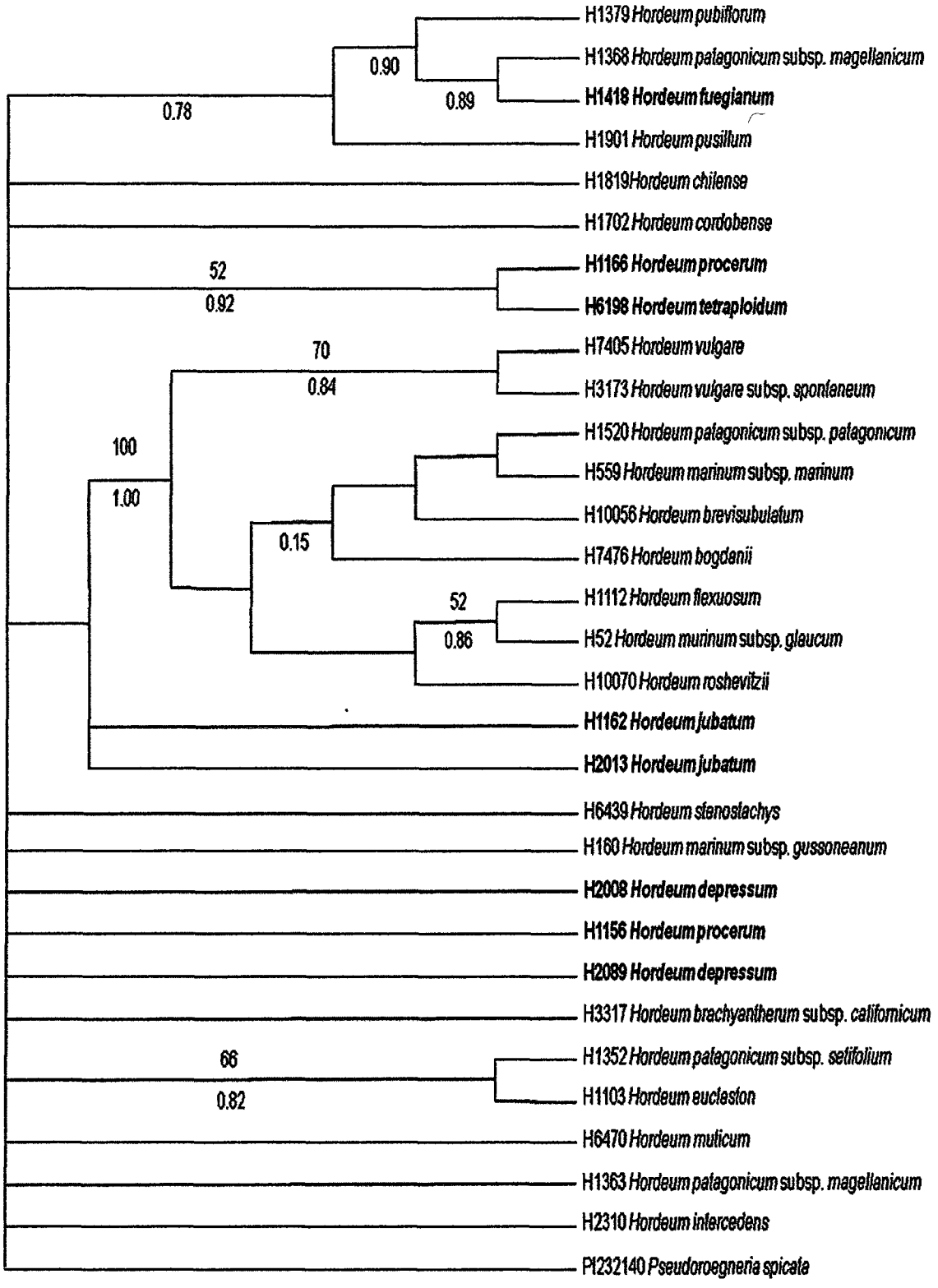
3.2 *Hordeum* Polyploids

3.2.1. Chloroplast DNA

trnD-trnT: Thirty trnD-trnT sequences were aligned, including twenty-two *Hordeum* diploid sequences and eight *Hordeum* polyploid sequences from six polyploids. Two obvious sequence types were observed, with distinctive sections of approximate 210 base pairs, similar as in Fig. 2. Of a total of 835 characters included in the final analysis, 633 characters were constant, 64 characters were parsimony-uninformative, and 141 characters were parsimony-informative. Phylogenetic analysis based on trnD-trnT region sequences was carried out using the MP and ML methods. A strict consensus tree from the 296 most parsimonious trees is shown in Fig. 6, with consistency index=0.818, retention index=0.938. Both the MP and ML analyses resulted in highly similar tree topologies consisting of two major clades. *H. fuegianum* is placed in the same clade as *H. pubiflorum, H. patagonicum* subsp. *magellanicum* and *H. pusillum* with an ALR of 0.78. *H. procerum* and *H. tetraploidum* form another clade, with bootstrap value of 52%, ALR of 0.92

Figure 6

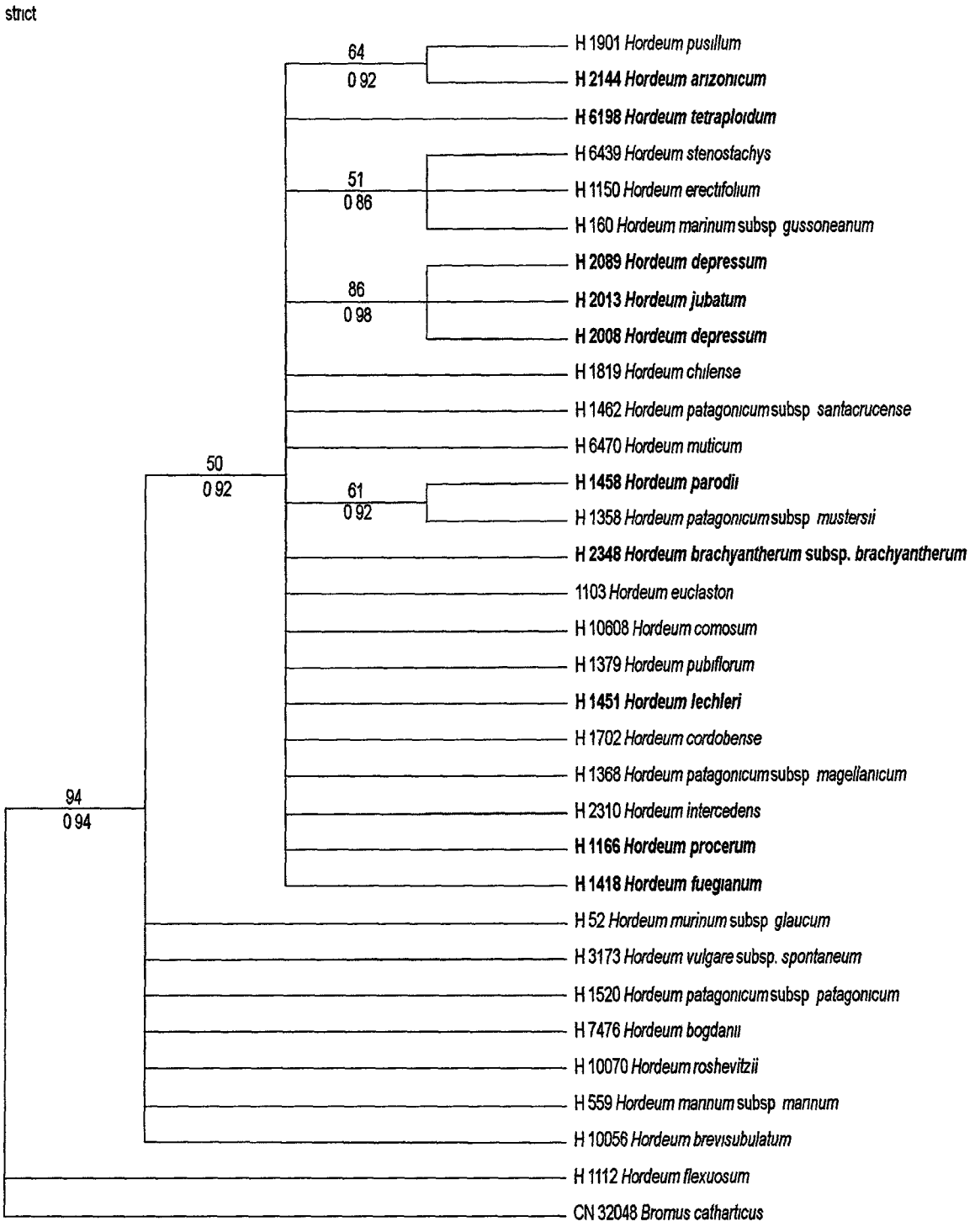
A strict consensus tree in the phylogenetic analysis of trnD-trnT dataset from 296 most parsimonious trees is shown, with consistency index=0.818, retention index=0.938. Thirty trnD-trnT sequences were aligned, including twenty-two *Hordeum* diploid sequences and eight *Hordeum* polyploid sequences from six polyploids. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. Species in bold are polyploids.


H1379 Hordeum pubiflorum
H1368 Hordeum patagonicum subsp. magellanicum
H1418 Hordeum fuegianum
H1901 Hordeum pusillum
H1819 Hordeum chilense
H1702 Hordeum cordobense
H1166 Hordeum procerum
H6198 Hordeum tetraploidum
H7405 Hordeum vulgare
H3173 Hordeum vulgare subsp. spontaneum
H1520 Hordeum patagonicum subsp. patagonicum
H559 Hordeum marinum subsp. marinum
H10056 Hordeum brevisubulatum
H7476 Hordeum bogdanii
H1112 Hordeum flexuosum
H52 Hordeum murinum subsp. glaucum
H10070 Hordeum roshevitzii
H1162 Hordeum jubatum
H2013 Hordeum jubatum
H6439 Hordeum stenostachys
H160 Hordeum marinum subsp. gussoneanum
H2008 Hordeum depressum
H1156 Hordeum procerum
H2089 Hordeum depressum
H3317 Hordeum brachyantherum subsp. californicum
H1352 Hordeum patagonicum subsp. setifolium
H1103 Hordeum euclesion
H6470 Hordeum muticum
H1363 Hordeum patagonicum subsp. magellanicum
H2310 Hordeum intercedens
PI232140 Pseudoroegneria spicata
0.90
0.89
0.78
52
0.92
70
0.84
100
1.00
0.15
52
0.86
66
0.82

rps16: Sequence comparisons among thirty-two sequences showed a low level of variation detected in the rps16 region. Of a total of 714 characters analyzed, 678 characters were constant, 244 characters were parsimony-uninformative, and only 12 characters were parsimony-informative. Maximum parsimonious analysis generated 42 trees. A strict consensus tree is shown in Fig. 7, with consistency index=0.929, retention index=0.875. The tree topologies from both MP and ML analyses showed great congruency with each other. *H. arizonicum* forms a clade with *H. pusillum* with a bootstrap value of 63%, and ALR of 0.92. Both accessions of *H. depressum* form a clade with *H. jubatum* with a high bootstrap value of 86% and ALR of 0.98. *H. parodii* is grouped with *H. patagonicum* subsp. *mustersii* with a bootstrap value of 61% and ALR of 0.92.

Figure 7

A strict consensus tree in the phylogenetic analysis of rps16 dataset from the 42 most parsimonious trees is shown, with consistency index=0.929, retention index=0.875. Thirty-two sequences were aligned, including twenty-two *Hordeum* diploid sequences and ten *Hordeum* polyploid sequences from nine polyploids. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. Species in bold are polyploids.

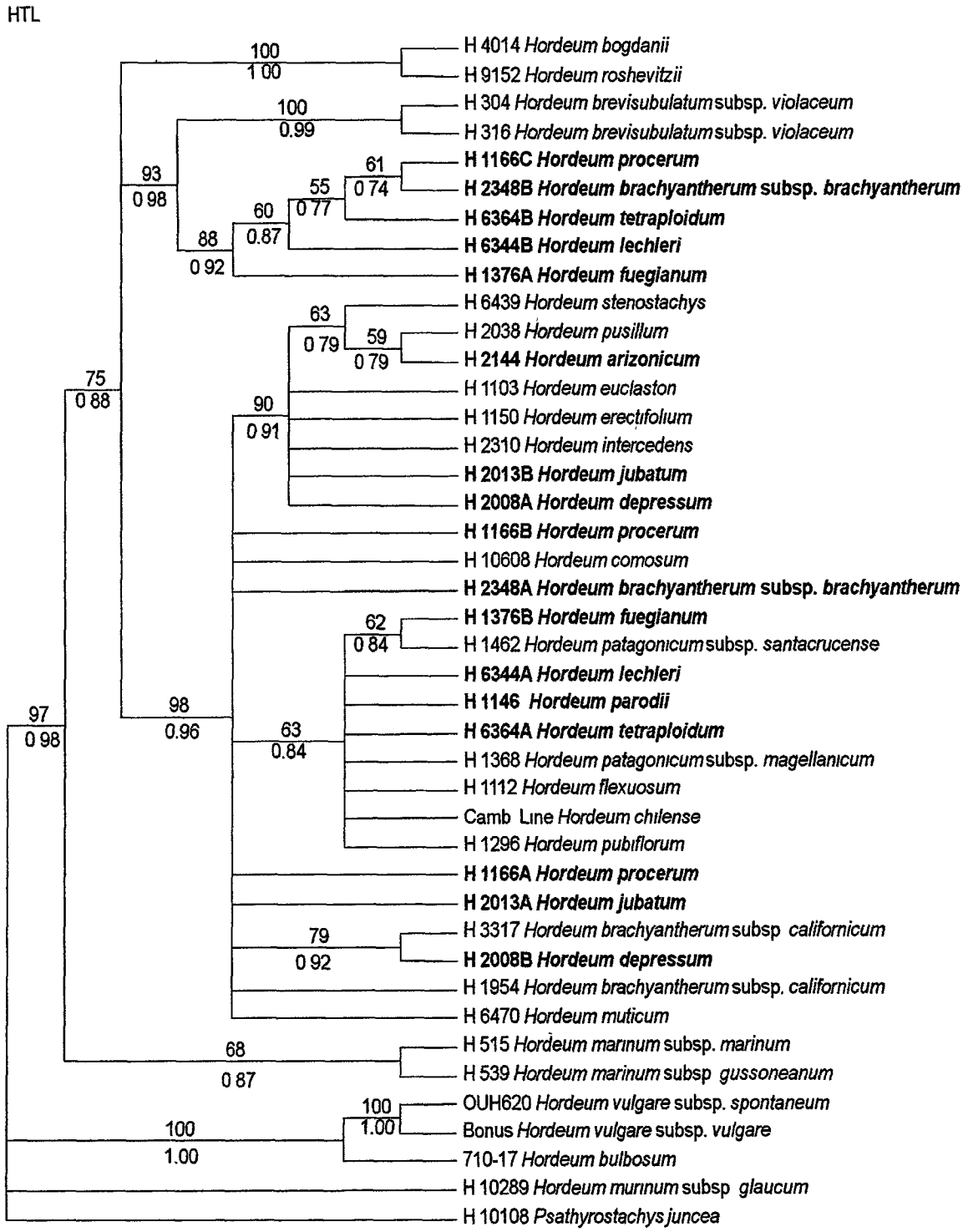

strict
64
0 92
51
0 86
86
0 98
50
0 92
61
0 92
94
0 94
H 1901 Hordeum pusillum
H 2144 Hordeum arizonicum
H 6198 Hordeum tetraploidum
H 6439 Hordeum stenostachys
H 1150 Hordeum erectifolium
H 160 Hordeum marinum subsp gussoneanum
H 2089 Hordeum depressum
H 2013 Hordeum jubatum
H 2008 Hordeum depressum
H 1819 Hordeum chilense
H 1462 Hordeum patagonicum subsp santacrucense
H 6470 Hordeum muticum
H 1458 Hordeum parodii
H 1358 Hordeum patagonicum subsp mustersii
H 2348 Hordeum brachyantherum subsp. brachyantherum
1103 Hordeum euclaston
H 10608 Hordeum comosum
H 1379 Hordeum pubiflorum
H 1451 Hordeum lechleri
H 1702 Hordeum cordobense
H 1368 Hordeum patagonicum subsp magellanicum
H 2310 Hordeum intercedens
H 1166 Hordeum procerum
H 1418 Hordeum fuegianum
H 52 Hordeum murinum subsp glaucum
H 3173 Hordeum vulgare subsp. spontaneum
H 1520 Hordeum patagonicum subsp patagonicum
H 7476 Hordeum bogdanii
H 10070 Hordeum roshevitzii
H 559 Hordeum marinum subsp marinum
H 10056 Hordeum brevisubulatum
H 1112 Hordeum flexuosum
CN 32048 Bromus catharticus

3.2.2. Nuclear genes

HTL: Forty-one HTL sequences were aligned, including seventeen polyploid sequences from nine different polyploid species. Of a total of 708 characters analyzed, 479 characters were constant, 119 characters were parsimony-uninformative, and 110 characters were parsimony-informative. MP and ML analyses based on the HTL sequences were carried out using *Psathyrostachy juncea* as the outgroup. The results of both MP and ML analyses showed high similarity in tree topologies. A strict consensus tree from the 299 most parsimonious trees is shown in Fig. 8, with consistency index of 0.866, retention index of 0.899. One copy from each of *H. procerum, H. brachyantherum* subsp. *brachyantherum, H. tetraploidum, H. lechleri* and *H. fuegianum* are grouped together with a high bootstrap value of 88% and ALR of 0.92. One copy from each of *H. jubatum, H. depressum* and *H. arizonicum* are placed in the same clade with some other *Hordeum* diploids with a high bootstrap value of 90% and ALR of 0.91. The other copy from each of *H. fuegianum, H. lechleri, H. tetraploidum* are placed in the same clade together with the only copy from *H. parodii,* with a bootstrap value of 63% and ALR of 0.84

Figure 8

A strict consensus tree in the phylogenetic analysis of HTL dataset from the 299 most parsimonious trees is shown, with consistency index=0.866, retention index=0.899. Forty-one HTL sequences were aligned, including seventeen polyploid sequences from nine different polyploid species. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. Species in bold are polyploids.

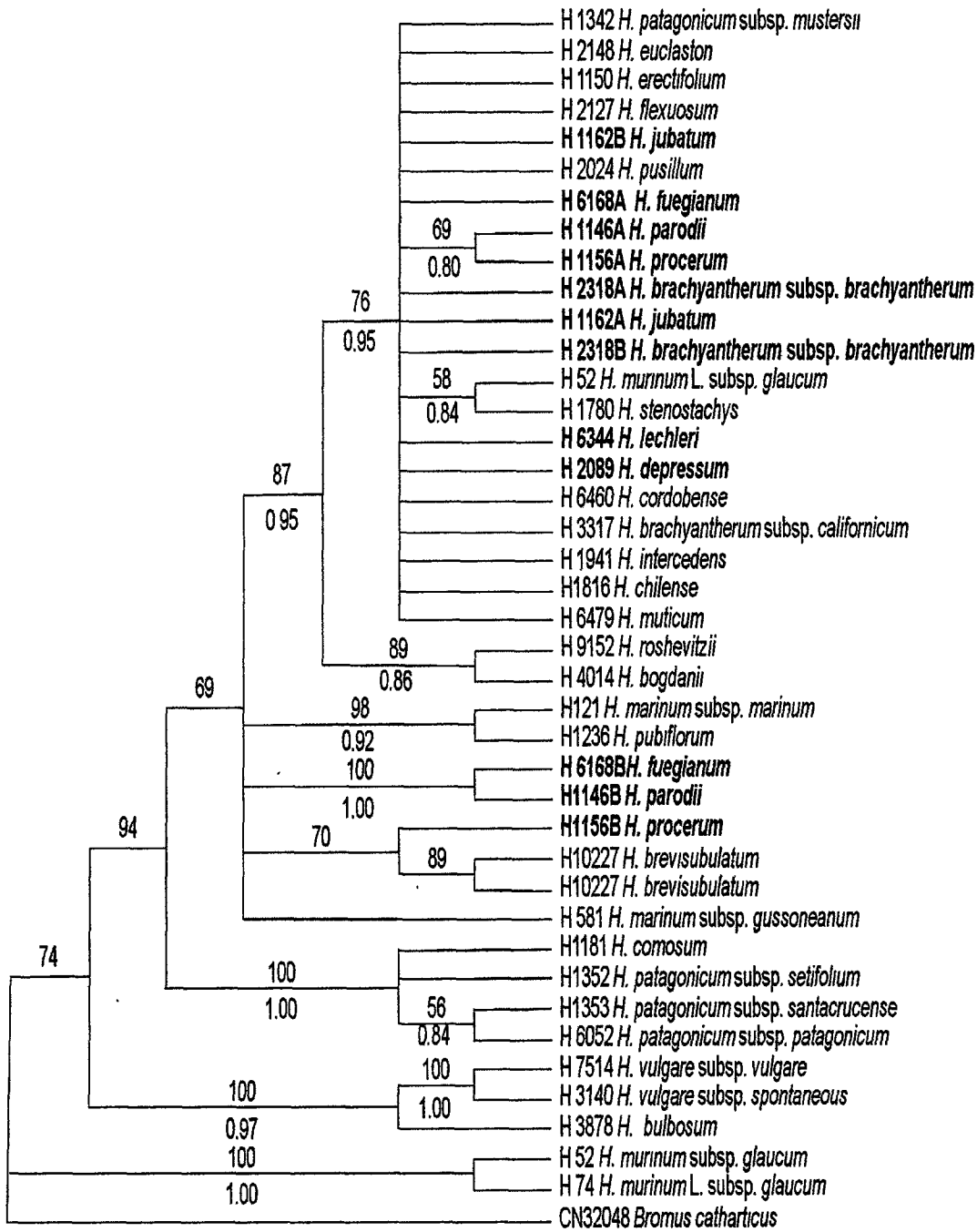

HTL
H 4014 Hordeum bogdanii
H 9152 Hordeum roshevitzii
H 304 Hordeum brevisubulatum subsp. violaceum
H 316 Hordeum brevisubulatum subsp. violaceum
H 1166C Hordeum procerum
H 2348B Hordeum brachyantherum subsp. brachyantherum
H 6364B Hordeum tetraploidum
H 6344B Hordeum lechleri
H 1376A Hordeum fuegianum
H 6439 Hordeum stenostachys
H 2038 Hordeum pusillum
H 2144 Hordeum arizonicum
H 1103 Hordeum euclaston
H 1150 Hordeum erectifolium
H 2310 Hordeum intercedens
H 2013B Hordeum jubatum
H 2008A Hordeum depressum
H 1166B Hordeum procerum
H 10608 Hordeum comosum
H 2348A Hordeum brachyantherum subsp. brachyantherum
H 1376B Hordeum fuegianum
H 1462 Hordeum patagonicum subsp. santacrucense
H 6344A Hordeum lechleri
H 1146 Hordeum parodii
H 6364A Hordeum tetraploidum
H 1368 Hordeum patagonicum subsp. magellanicum
H 1112 Hordeum flexuosum
Camb Line Hordeum chilense
H 1296 Hordeum pubiflorum
H 1166A Hordeum procerum
H 2013A Hordeum jubatum
H 3317 Hordeum brachyantherum subsp californicum
H 2008B Hordeum depressum
H 1954 Hordeum brachyantherum subsp. californicum
H 6470 Hordeum muticum
H 515 Hordeum marinum subsp. marinum
H 539 Hordeum marinum subsp gussoneanum
OUH620 Hordeum vulgare subsp. spontaneum
Bonus Hordeum vulgare subsp. vulgare
710-17 Hordeum bulbosum
H 10289 Hordeum murinum subsp glaucum
H 10108 Psathyrostachys juncea

Rpb2: Forty rpb2 sequences were aligned, including twelve polyploid sequences from seven different polyploid species. Of a total of 687 characters analyzed, 426 characters were constant, 79 characters were parsimony-uninformative, and 182 characters were parsimony-informative. MP and ML analyses based on the rpb2 sequences were carried out using *Bromus catharticus* as the outgroup. The results of both MP and ML analyses showed high similarity in tree topologies. A strict consensus tree from the 444 most parsimonious trees is shown in Fig. 9, with consistency index of 0.777, retention index of 0.902. All the polyploids have at least one copy grouped in the same clade with I genome diploids, with a bootstrap value of 76% and ALR value of 0.96. Within this clade, one copy from each of *H. parodii* and *H. procerum* were grouped together with a bootstrap value of 69% and ALR value of 0.80. In addition, both copies of each of *H. jubatum* and *H. brachyantherum* subsp. *brachyantherum* were grouped in the same clade as well. The other copy of the three polyploids *H. parodii, H. procerum* and *H. fuegianum* were grouped outside this clade. Of the three, the copy of *H. fuegianum* and *H. parodii* were grouped together with a high bootstrap value of 100% and ALR value of 1.00

Figure 9

A strict consensus tree in the phylogenetic analysis of rpb2 dataset from the 444 most parsimonious trees is shown, with consistency index=0.777, retention index=0.902. Forty rpb2 sequences were aligned, including twelve polyploid sequences from seven different polyploid species. The tree topologies from both MP and ML analyses showed great congruency with each other. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate likelihood ratio test (ALR) values from ML analysis. Species in bold are polyploids.

Strict


H 1342 H. patagonicum subsp. mustersii
H 2148 H. euclaston
H 1150 H. erectifolium
H 2127 H. flexuosum
H 1162B H. jubatum
H 2024 H. pusillum
H 6168A H. fuegianum
69
H 1146A H. parodii
0.80
H 1156A H. procerum
H 2318A H. brachyantherum subsp. brachyantherum
76
H 1162A H. jubatum
0.95
H 2318B H. brachyantherum subsp. brachyantherum
58
H 52 H. murinum L. subsp. glaucum
0.84
H 1780 H. stenostachys
H 6344 H. lechleri
87
H 2089 H. depressum
0 95
H 6460 H. cordobense
H 3317 H. brachyantherum subsp. californicum
H 1941 H. intercedens
H1816 H. chilense
H 6479 H. muticum
89
H 9152 H. roshevitzii
0.86
H 4014 H. bogdanii
69
98
H121 H. marinum subsp. marinum
0.92
H1236 H. pubiflorum
100
H 6168BH. fuegianum
1.00
H1146B H. parodii
94
70
H1156B H. procerum
89
H10227 H. brevisubulatum
H10227 H. brevisubulatum
H 581 H. marinum subsp. gussoneanum
74
H1181 H. comosum
100
H1352 H. patagonicum subsp. setifolium
1.00
56
H1353 H. patagonicum subsp. santacrucense
0.84
H 6052 H. patagonicum subsp. patagonicum
100
H 7514 H. vulgare subsp. vulgare
100
1.00
H 3140 H. vulgare subsp. spontaneous
0.97
H 3878 H. bulbosum
100
H 52 H. murinum subsp. glaucum
1.00
H 74 H. murinum L. subsp. glaucum
CN32048 Bromus catharticus

DMC1: A total of nine *Hordeum* polyploidy species were amplified with the TDMC 1e10/TDMC 1e15R primer pair. The size of amplified fragments ranged from ~950bp to ~1050bp. Eight out of the nine polyploid species were found to have at least two copies for the DMC1 gene, while only one copy out of ten clones from *Hordeum lechleri* (Accession H1451) was identified. Sequence comparisons revealed three large insertions/deletions (indels) (Fig. 10). The largest indel occurred at position 971 in one copy sequence from polyploid species *H. jubatum*, *H. tetraploidum* and *H. fuegianum,* showing an 82 bp insertion compared to diploid *Hordeum* species (Fig. 10A). BLAST search against this indel found that it belonged to a miniature inverted-repeat transposable element (MITE), more specifically *Stowaway* element, as reported by Petersen and Seberg (2000). This element contains 36 bp terminal inverted repeats (TIR). The target site is a TA short sequence (Wessler *et al.*, 1995; Petersen and Seberg, 2000), and the *Stowaway* element is flanked by a direct TA repeat, which should be the result of transposition. Another indel occurred at position 292 with a 24 bp insertion found only in one copy sequence from polyploid *H. procerum* and *H. cordobense*, which is $(GCCACGTAAATTTACAAGTTGATT)_2$ repeat (Fig. 10B). The last indel occurred at position 1167 with a 15 bp deletion found in *H. procerum, H. arizonicum, H. brachyantherum* subsp. *brachyantherum, H. depressum* and *H. brachyantherum* subsp. *californicum* (Fig. 10C).

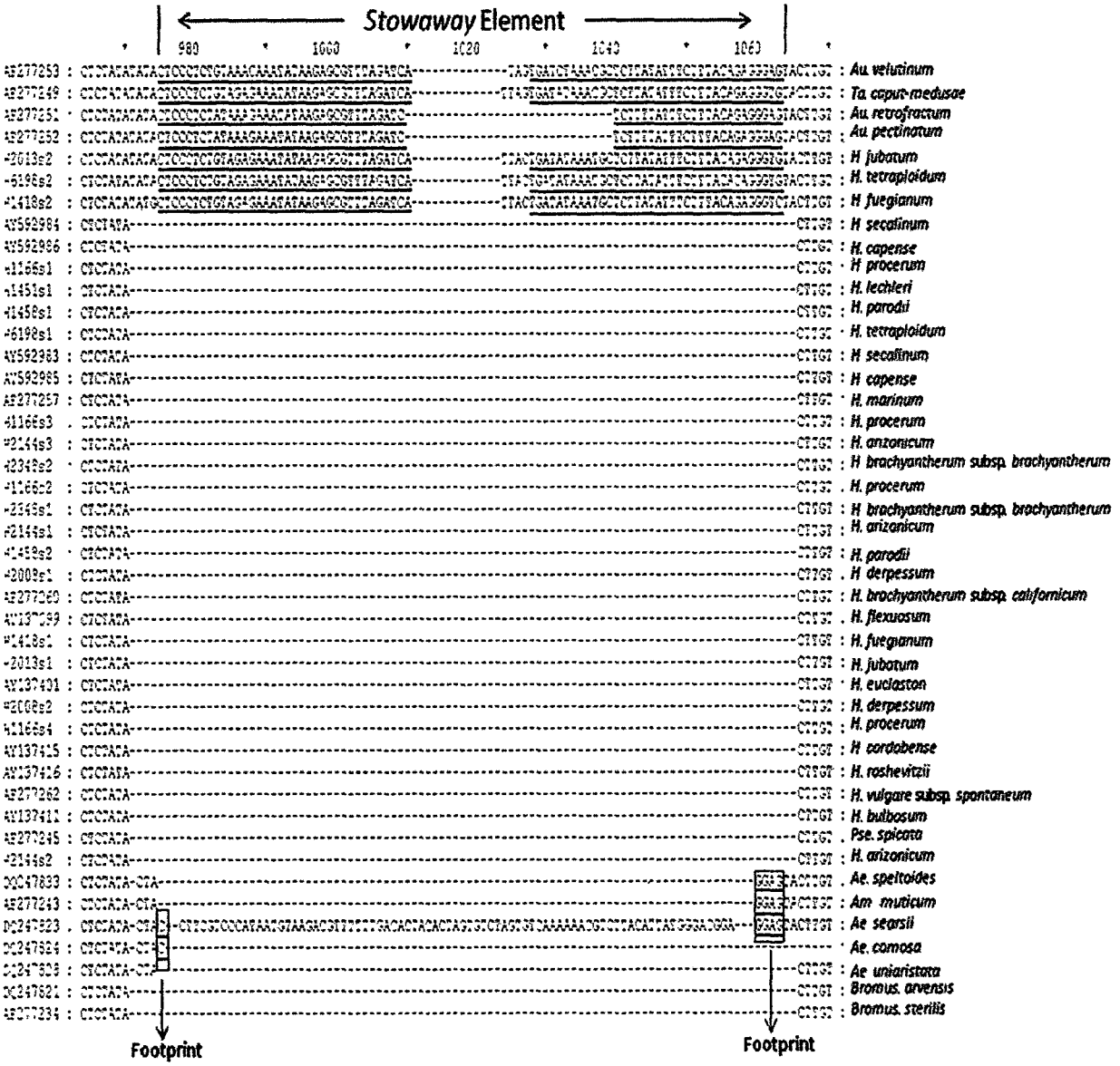
In order to investigate the putative origins of the polyploid *Hordeum* species, we constructed the phylogeny for only *Hordeum* species. Two *Bromus* species were used as outgroups. The cladistic parsimony analysis yielded the 290 most parsimonious

trees with a consistency index (CI) of 0.848 and retention index (RI) of 0.925. One copy from *H. jubatum, H. tetraploidum, H. fuegianum* and *H. arizonicum* formed a monophyletic group with bootstrap support value of 69%, which is sister to the remaining *Hordeum* species (Fig. 11). The first three species were grouped together with 100% bootstrap support value, indicating their common origin. One copy of each of *H. lechleri, H. parodii, H. tetraploidum* and *H. procerum* species were grouped together with 100% bootstrap support value. This group was included in the same clade with diploid I, Xa and Xu genome species, indicating that these four sequences originated from *Hordeum* species. In addition, polyploid species *H. arizonicum, H. procerum, H. brachyantherum* subsp. *brachyantherum* and *H. depressum* were grouped together with diploid species *H. brachyantherum* subsp. *californicum*, with a bootstrap support value of 95%.

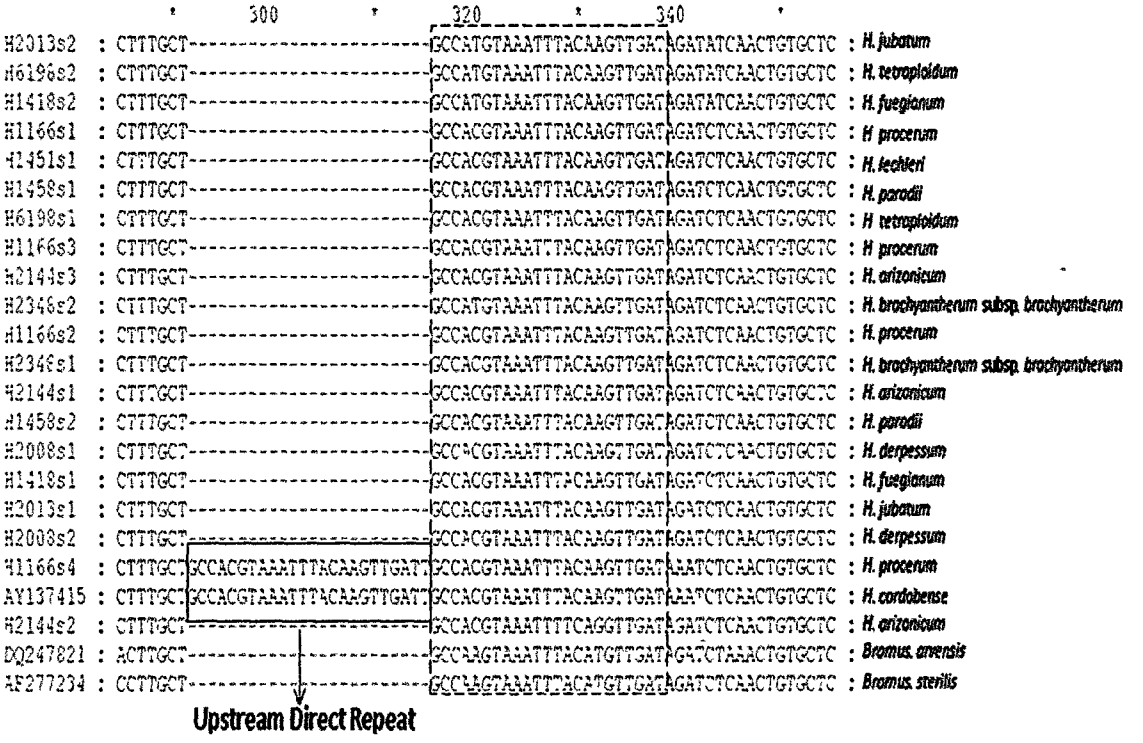
Figure 10

Partial alignment of the DMC1 sequences from *Hordeum* diploids and polyploids were displayed here. Three major indels were detected. Fig. 10A displayed the largest indel occurred at position 971 in one copy sequence from polyploid species *H. jubatum*, *H. tetraploidum* and *H. fuegianum*, showing an 82 bp insertion compared to diploid *Hordeum* species. This element contains 36 bp terminal inverted repeats (TIR). The target site is a TA short sequence. Fig. 10B displayed the second indel occurred at position 292 with a 24 bp insertion found only in one copy sequence from polyploid *H. procerum* and *H. cordobense*, which is $(GCCACGTAAATTTACAAGTTGATT)_2$ repeat. Fig. 10C displayed the last indel occurred at position 1167 with a 15 bp deletion found in *H. procerum, H. arizonicum, H. brachyantherum* subsp. *brachyantherum, H. depressum* and *H. brachyantherum* subsp. *californicum*.

A


Stowaway Element
980
1000
1020
1040
1060
Au. velutinum
Ta. caput-medusae
Au. retrofractum
Au. pectinatum
H. jubatum
H. tetraploidum
H. fuegianum
H. secalinum
H. capense
H. procerum
H. lechleri
H. parodii
H. tetraploidum
H. secalinum
H. capense
H. marinum
H. procerum
H. arizonicum
H. brachyantherum subsp. brachyantherum
H. procerum
H. brachyantherum subsp. brachyantherum
H. arizonicum
H. parodii
H. derpessum
H. brachyantherum subsp. californicum
H. flexuosum
H. fuegianum
H. jubatum
H. euclaston
H. derpessum
H. procerum
H. cordobense
H. roshevitzii
H. vulgare subsp. spontaneum
H. bulbosum
Pse. spicata
H. arizonicum
Ae. speltoides
Am. muticum
Ae. searsii
Ae. comosa
Ae. uniaristata
Bromus. arvensis
Bromus. sterilis
Footprint
Footprint

B


300
320
340
H2013s2 : CTTTGCT----------GCCATGTAAATTTACAAGTTGATAGATATCAACTGTGCTC : H. jubatum
H6196s2 : CTTTGCT----------GCCATGTAAATTTACAAGTTGATAGATATCAACTGTGCTC : H. tetraploidum
H1418s2 : CTTTGCT----------GCCATGTAAATTTACAAGTTGATAGATATCAACTGTGCTC : H. fuegianum
H1166s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H procerum
H1451s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. lechleri
H1458s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. parodii
H6198s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H tetraploidum
H1166s3 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H procerum
H2144s3 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. arizonicum
H2346s2 : CTTTGCT----------GCCATGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. brachyantherum subsp. brachyantherum
H1166s2 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. procerum
H2346s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. brachyantherum subsp. brachyantherum
H2144s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. arizonicum
H1458s2 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. parodii
H2008s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. derpessum
H1418s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. fuegianum
H2013s1 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. jubatum
H2008s2 : CTTTGCT----------GCCACGTAAATTTACAAGTTGATAGATCTCAACTGTGCTC : H. derpessum
H1166s4 : CTTTGCTGCCACGTAAATTTACAAGTTGATTGCCACGTAAATTTACAAGTTGATAAATCTCAACTGTGCTC : H. procerum
AY137415 : CTTTGCTGCCACGTAAATTTACAAGTTGATTGCCACGTAAATTTACAAGTTGATAAATCTCAACTGTGCTC : H. cordobense
H2144s2 : CTTTGCT----------GCCACGTAAATTTTCAGGTTGATAGATCTCAACTGTGCTC : H. arizonicum
DQ247821 : ACTTGCT----------GCCAAGTAAATTTACATGTTGATAGATCTAAACTGTGCTC : Bromus. anensis
AF277234 : CCTTGCT----------GCCAAGTAAATTTACATGTTGATAGATCTCAACTGTGCTC : Bromus. sterilis
Upstream Direct Repeat

C

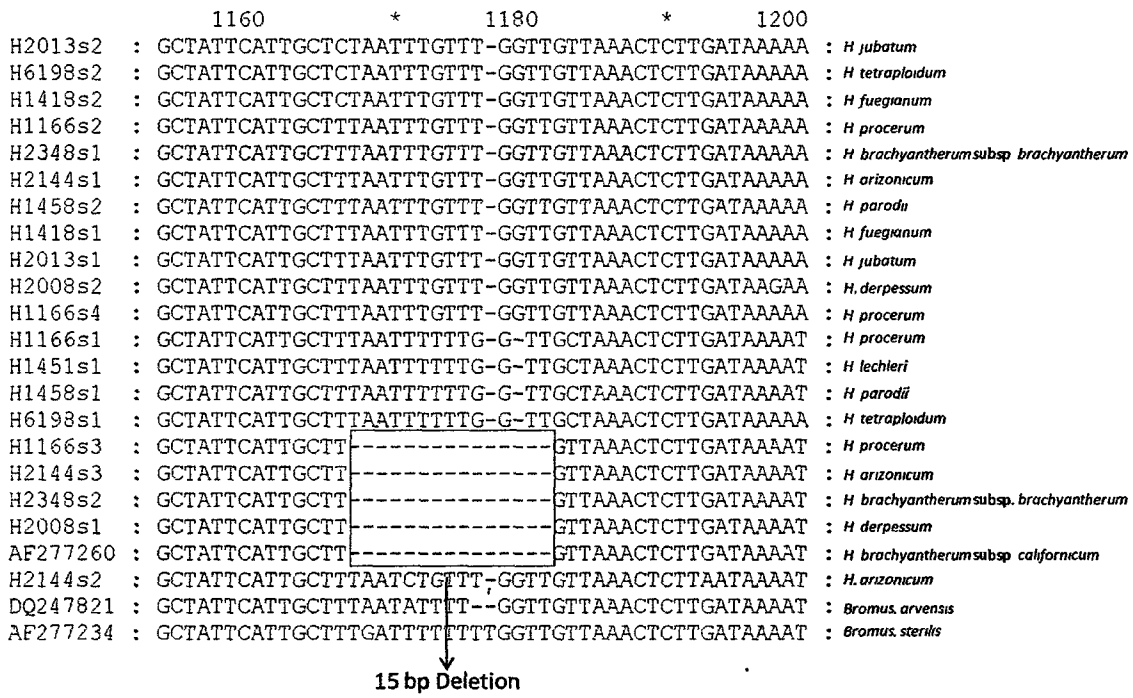
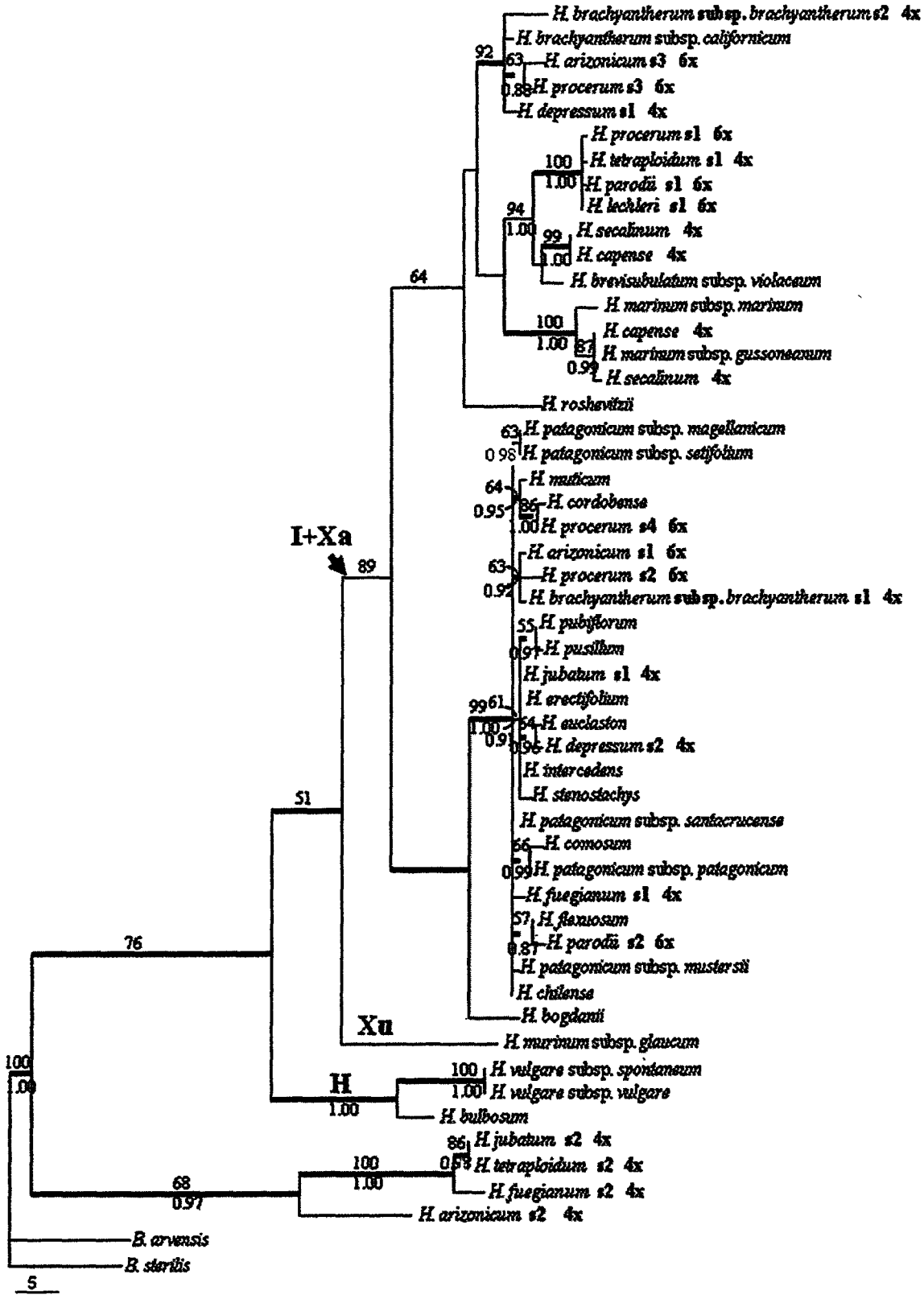

1160 * 1180 * 1200
H2013s2 : GCTATTCATTGCTCTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H jubatum
H6198s2 : GCTATTCATTGCTCTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H tetraploidum
H1418s2 : GCTATTCATTGCTCTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H fuegianum
H1166s2 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H procerum
H2348s1 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H brachyantherum subsp brachyantherum
H2144s1 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H arizonicum
H1458s2 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H parodii
H1418s1 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H fuegianum
H2013s1 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H jubatum
H2008s2 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAGAA : H. derpessum
H1166s4 : GCTATTCATTGCTTTAATTTGTTT-GGTTGTTAAACTCTTGATAAAAA : H procerum
H1166s1 : GCTATTCATTGCTTTAATTTTTTG-G-TTGCTAAACTCTTGATAAAAT : H procerum
H1451s1 : GCTATTCATTGCTTTAATTTTTTG-G-TTGCTAAACTCTTGATAAAAT : H lechleri
H1458s1 : GCTATTCATTGCTTTAATTTTTTG-G-TTGCTAAACTCTTGATAAAAT : H parodii
H6198s1 : GCTATTCATTGCTTTAATTTTTTG-G-TTGCTAAACTCTTGATAAAAA : H tetraploidum
H1166s3 : GCTATTCATTGCTT----------GTTAAACTCTTGATAAAAT : H procerum
H2144s3 : GCTATTCATTGCTT----------GTTAAACTCTTGATAAAAT : H arizonicum
H2348s2 : GCTATTCATTGCTT----------GTTAAACTCTTGATAAAAT : H brachyantherum subsp. brachyantherum
H2008s1 : GCTATTCATTGCTT----------GTTAAACTCTTGATAAAAT : H derpessum
AF277260 : GCTATTCATTGCTT----------GTTAAACTCTTGATAAAAT : H brachyantherum subsp californicum
H2144s2 : GCTATTCATTGCTTTAATCTGTTT-GGTTGTTAAACTCTTAATAAAAT : H. arizonicum
DQ247821 : GCTATTCATTGCTTTAATATTTT--GGTTGTTAAACTCTTGATAAAAT : Bromus arvensis
AF277234 : GCTATTCATTGCTTTGATTTTTTTTGGTTGTTAAACTCTTGATAAAAT : Bromus sterilis
15 bp Deletion

Figure 11

A strict consensus tree in the phylogenetic analysis of DMC1 dataset from the 280 most parsimonious trees is shown, with consistency index=0.848, retention index=0.925. Fifty-two DMC1 sequences were aligned, including twenty polyploid sequences from nine different polyploid species obtained in our study. Numbers above branches are bootstrap values from MP analysis and numbers below branches are approximate Bayesian values. Species in bold are polyploids.


H. brachyantherum subsp. brachyantherum s2 4x
H. brachyantherum subsp. californicum
H. arizonicum s3 6x
H. procerum s3 6x
H. depressum s1 4x
H. procerum s1 6x
H. tetraploidum s1 4x
H. parodii s1 6x
H. lechleri s1 6x
H. secalinum 4x
H. capense 4x
H. brevisubulatum subsp. violaceum
H. marinum subsp. marinum
H. capense 4x
H. marinum subsp. gussoneanum
H. secalinum 4x
H. roshevitzii
H. patagonicum subsp. magellanicum
H. patagonicum subsp. setifolium
H. muticum
H. cordobense
H. procerum s4 6x
I+Xa
H. arizonicum s1 6x
H. procerum s2 6x
H. brachyantherum subsp. brachyantherum s1 4x
H. pubiflorum
H. pusillum
H. jubatum s1 4x
H. erectifolium
H. euclaston
H. depressum s2 4x
H. intercedens
H. stenostachys
H. patagonicum subsp. santacrucense
H. comosum
H. patagonicum subsp. patagonicum
H. fuegianum s1 4x
H. flexuosum
H. parodii s2 6x
H. patagonicum subsp. mustersii
H. chilense
H. bogdanii
Xu
H. marinum subsp. glaucum
H. vulgare subsp. spontaneum
H
H. vulgare subsp. vulgare
H. bulbosum
H. jubatum s2 4x
H. tetraploidum s2 4x
H. fuegianum s2 4x
H. arizonicum s2 4x
B. arvensis
B. stellis
5

4. Discussion

4.1. Interpret the incongruence between nuclear data and chloroplast data

Incongruences between nuclear and chloroplast phylogenies were found for: 1) the position of Xa genome species *H. marinum* subsp. *gussoneanum*. 2) the position of the three I genome group species *H, brevisubulatum, H. roshevitzii,* and *H. bogdanii.* 3) the position of I genome group species *H. flexuosum.*

The major incongruence is that our nuclear data support the monophyletic group of *H. marinum* subspecies while our chloroplast data sets place the *H. marinum* subsp. *gussoneanum* in another clade with American I genome species (Fig. 3, 4, 5). This discrepancy in placing *H. marinum* subspecies is consistent with previous studies. Previous nuclear data (Blattner 2004; Jakob *et al.*, 2007; Kakeda *et al.*, 2009; Komatsuda *et al.* 2001; Petersen and Seberg 2004; Sun *et al.* 2009) grouped *H. marinum* subspecies together (Xa genome group) as the sister group to I genome species. On the contrary, chloroplast data consistently placed *H. marinum* subsp. *gussoneanum* in the clade with I genome species and *H. marinum* subsp. *marinum* in another clade with other genome species (Provan *et al.*, 1999; Doebley *et al.*, 1992; Nishikawa *et al.*, 2002; Petersen and Seberg, 2003; Jakob and Blattner, 2006). There are three possible explanations for such discrepancy: incomplete lineage sorting, hidden paralogy, and horizontal gene transfer. It has been demonstrated that HTL is

the single copy nuclear gene in *Hordeum* species (Kakeda *et al.*, 2008), and therefore the possibility of hidden paralogy as the cause of the discrepancy in our study can be ruled out. Also, Petersen and Seberg (2003), as well as Jakob and Blattner (2006), argued that it is unlikely that horizontal gene transfer leads to this particular incongruence, based on two reasons. First, crossing experiments (von Bothmer *et al.*, 1986) showed a high resistance of *H. marinum* to natural hybridization. Second, even if *H. marinum* subsp. *gussoneanum* is able to undergo successful backcrossing, it is unlikely for it to remove all the other traits but the "wrong" traits of plastid donor, which must be American-type plastid, through repeated backcrossing. Therefore, incomplete lineage sorting seems to be the reasonable explanation for such incongruence. Our chloroplast phylogeny results supported their studies by showing similar tree topologies inferred by two different chloroplast regions, and thus further strengthened such conclusion.

Another incongruence is that both of trnD-trnT and rps16 phylogenies grouped Euraisan I genome species *H. brevisubulatum, H. roshevitzii,* and *H. bogdanii* with Xa species (*H. marinum* subsp. *marinum*), H species (*H. vulgare*) and Xu species (*H. murinum*) together, indicating they share a similar plastid type (Fig. 2), while the HTL phylogeny grouped these three Eurasian I genome species with American I genome species (Fig. 3, 4, 5). The HTL phylogeny is supported by previous nuclear studies (Blattner 2004; Petersen and Seberg 2004, 2006; Jakob *et al.*, 2007; Kakeda *et al.*, 2009; Sun *et al.* 2009), suggesting these three Eurasian I genome species are in a closer relationship with American I genome diploid species. However, the separation of I genome species in both chloroplast phylogenies actually corresponds well with

the geographic distribution of *Hordeum* species, because these three I genome species are all distributed in Central and East Asia, closer to other genome group species distributed in western Eurasia, while all other I genome species share a common distribution in American continents. This assumption is also strongly supported by the fact that all the Eurasian species share the same chloroplast type in trnD-trnT sequence alignment while all the American I genome species share the other chloroplast type (Fig. 2).

The third incongruence resulted from the different placements of *H. flexuosum*. Both chloroplast and nuclear phylogenies from previous studies have placed *H. flexuosum* in the American I genome clade, which is also supported by the HTL phylogeny in our result (Fig. 5). However, our chloroplast data are inconsistent with previous chloroplast studies (Provan *et al.*, 1999; Doebley *et al.*, 1992; Nishikawa *et al.*, 2002; Petersen and Seberg, 2003; Jakob and Blattner, 2006). In the present study, trnD-trnT and rps16 phylogenies suggested *H. flexusosum* having a distant plastid type from the remaining American species (Figure 3, 4).

4.2 Phylogeny of *Hordeum* diploids

Due to a low evolutionary rate, the phylogenetic result from the rps16 region provides a poor resolution of the phylogeny of *Hordeum* species. It only suggests close relatedness among American continental species with a bootstrap value of 50% and ATR of 0.907. Phylogeny of the trnD-trnT region provides a higher resolution because of its higher level of sequence variation. It strongly supports the close

relationship_of American species *H. flexuosum* and all Eurasian species with a bootstrap value of 100% and ATR of 1.00. In addition, the trnD-trnT phylogeny indicates *H. cordobense, H. patagonicum* subsp. *setifolium, H. brachyantherum* subsp. *californicum* and *H. marrinum* subsp. *guessoneanum* share a similar chloroplast haplotype (bootstrap value of 65% and ATR of 0.939), which provides a higher resolution among I genome new world species than some previous studies (Komatsuda, 1999; Petersen and Seberg, 2003; Sun *et al.*, 2009).

Phylogenetic analysis of HTL sequences highly corresponds with the classification of four genome groups of *Hordeum* species, with the clade of I/ Xa (bootstrap value of 99%, ATR of 0.99) and the clade of H/Xu (bootstrap value of 86%, ATR of 0.74). Furthermore, it successfully separates I and Xa group species with a bootstrap value of 74%, ATR of 0.84, and a bootstrap value of 89%, ATR of 0.93 respectively. In addition, it also separates H genome species from Xu species with a bootstrap value of 100% and ATR of 1.00. This illustrates the efficiency of HTL as a tool in solving the phylogeny of the genus *Hordeum.* Because the HTL phylogeny of eleven *Hordeum* species has been discussed by Kakeda *et al.* (2009), here we extend the discussion to the phylogeny of newly added *Hordeum* diploids. By adding nine more species to the result of Kakeda *et al* (2009), our HTL phylogeny is even able to provide a close relationship of *H. intercedens, H. erectifilium, H. pusillum, H. stenostachys* and *H. euclaston*, with a bootstrap value of 94% and ATR of 0.98. Petersen and Seberg (2009) reached a similar conclusion using nucleotide sequence data of the xylose isomerase (XYL). This result is also consistent with Blattner's

finding (2004, 2006) supported by rDNA ITS sequences and single copy nuclear genes DMC1 and EF-G.

4.3 The Origins of *Hordeum* Polyploids

4.3.1 Origin of Tetraploid Species

The nature of polyploidy of the annual tetraploid *H. depressum,* distributed in the western United States, has been a matter of debate. *H. depressum* used to be considered to have an autoploid origin, based on its high autosyndetic pairing (Sakamoto, 1974; Petersen, 1991). However, FISH and RFLP analysis suggested a segmental alloploid origin of *H. depressum*, with *H. brachyantherum* subsp. *californicum* as one parent and either *H. pussillum* or *H. intercedens* as the other parent (Taketa *et al.*, 2005), which was consistent with the results of Baum and Bailey (1988) and Covas (1949). Chloroplast DNA suggested *H. brachyantherum* subsp. *californicum* as the maternal parent of *H. depressum* (Doebley *et al*, 1992; Jakob and Blattner 2006). Two distinct DMC1 copies were discovered here, and phylogenetic analysis separated the two copies into different groups (Fig. 11), suggesting the allopolyploid origin of *H. depressum*. One copy was grouped with *H. brachyantherum* subsp. *californicum*, with 95% strong bootstrap support, which further confirms *H. brachyantherum* subsp. *californicum* as one genome donor of *H. depressum* (Covas, 1949; Baum and Bailey, 1988; Doebley *et al.*, 1992; Taketa *et al.*, 2005; Jakob and Blattner 2006;). Another copy of sequence formed a group with six diploid species (*H. erectifilium*, *H. euclaston*, *H. intercedens*, *H. pubiflorum*, *H.*

pusillum and *H. stenostachys*) with weak bootstrap support (52%). It seems that *H. depressum* is close to diploid *H. euclaston* with 58% support value (Fig. 11), but our results did not contradict the suggestion of Blattner (2004) that *H. intercedens* is the other diploid genome donor to *H. depressum*. Geographical distribution of *H. depressum* only overlaps with one of these six diploid species, *H. intercedens*. Taking the geographical distribution into consideration, *H. intercedens* is more likely another genome donor to the tetraploid *H. depressum*. Two distinct copies of HTL sequences were also discovered in the present study (Fig. 8). One copy was grouped together with *H. brachyantherum* subsp. *californicum,* with a bootstrap value of 79% and ALR of 0.92. Another copy was placed in the same clade with polyploids *H. jubatum, H. arizonicum* and diploids including *H. stenostachys, H. pusillum, H. euclaston, H. erectifilium* and *H. intercedens.* The positions of both copies of HTL sequences greatly agree with the results of the DMC1 phylogeny, further indicating that *H. brachyantherum* subsp. *californicum* is very likely one genome donor of *H. depressum,* and *H. intercedens* or *H. euclaston* could be the other genome donor. However, only one copy of rpb2 sequences from *H. depressum* was discovered in this study, and this copy together with most "new world" diploids and other polyploids formed a major clade with a bootstrap value of 76% and ALR of 0.95 (Fig. 9). Though the resolution of the rpb2 phylogeny is not high enough to infer the genome donors of *H. depressum,* it does not contradict with other nuclear datasets in the present study because the possible genome donors *H. brachyantherum* subsp. *californicum, H. intercedens* and *H. euclaston* were in the same clade with *H. depressum.* Two accessions of *H. depressum* were used in both chloroplast

phylogenies (Fig. 6, 7). However, neither of them is able to infer the maternal genome donor of *H. depressum.* In rps16 phylogeny, both accessions of *H. depressum* were in the same group with *H. jubatum,* with a high bootstrap value of 86% and ALR of 0.98, indicating they probably share the same maternal genome donor (Fig. 7). Future research employing other chloroplast genes is needed to investigate the maternal genome donor for *H. depressum.*

The other four tetraploid species, *H. jubatum, H. tetraploidum, H. fuegianum* and *H. brachyantherum* subsp. *Brachyantherum,* are all perennial species. Karyotype analysis suggested that *H. brachyantherum* subsp. *californicum* is one of the ancestors for *H. brachyantherum* subsp. *brachyantherum* and *H. jubatum* (Linde-Laursen *et al.,* 1995) which was supported by RFLP and FISH pattern of Taketa *et al* (2005). Chloroplast DNA data suggested *H. brachyantherum* subsp. *californicum* as the maternal genome donor to *H. brachyantherum* subsp. *brachyantherum* (Nishikawa *et al.,* 2002: Jakob and Blattner 2006). In the present study, our DMC1 sequence data revealed a strong relationship between *H. brachyantherum* subsp. *californicum* and *H. brachyantherum* subsp. *brachyantherum,* with evidence with 92% bootstrap support (Fig. 11), and further confirmed that *H. brachyantherum* subsp. *californicum* is one of the ancestors of *H. brachyantherum* subsp. *brachyantherum.* However, there was no firm evidence to infer the other parent from the DMC1 result. Two distinct copies of HTL sequences from *H. brachyantherum* subsp. *brachyantherum* were discovered (Fig. 8). One copy was placed in a large group with I genome "new world" species, including *H. brachyantherum* subsp. *californicum,*

and many other polyploids, with a high bootstrap value of 98% and ALR of 0.91. However, the resolution of this clade is not enough to conclude that *H. brachyantherum* subsp. *californicum* is one genome donor of *H. brachyantherum* subsp. *brachyantherum.* The other HTL copy of *H. brachyantherum* subsp. *brachyantherum* is grouped together with *H. procerum, H. tetraploidum, H. lechleri* and *H. fuegianum*, with a high bootstrap value of 88% and ALR of 0.92. This clade is grouped with both accessions of *H. brevisubulatum* subsp. *violaceum*, indicating *H. brevisubulatum* subsp. *violaceum* might be the possible other parent of *H. brachyantherum* subsp. *brachyantherum.* In addition, of the four polyploids, *H. brachyantherum* subsp. *brachyantherum* is most closely related with *H. procerum,* suggesting a common ancestor. This is also supported in the DMC1 phylogeny, in which both copies of the two polyploids were grouped together. In the rpb2 phylogeny, two distinct copies of *H. brachyantherum* subsp. *brachyantherum* were also discovered. However, due to a relatively low variation in *Hordeum* polyploids, we could not infer any particular diploid as the genome donor to *H. brachyantherum* subsp. *brachyantherum.* Both copies of *H. brachyantherum* subsp. *brachyantherum* were grouped in a large clade with I genome "new world" diploid *Hordeum* species, including the potential genome donor *H. brachyantherum* subsp. *californicum,* with a high bootstrap value of 98% and ALR of 0.96. This does not contradict with the other two nuclear datasets. Taketa *et al* (2005) suggested the Old World species *H. roshevitzii* should be the other parent, based on the FISH pattern and RFLP profiles. Blattern (2004) also employed rDNA ITS sequences and reached a similar conclusion of *H. roshevitzii* as the other parent. However, our nuclear datasets did not favor the

suggestion of *H. roshevitzii* as the other parent for *H. brachyantherum* subsp. *brachyantherum.* In the rps16 phylogeny based on chloroplast DNA, *H. brachyantherum* subsp. *brachyantherum* was also grouped in a large clade with I genome "new world" diploid *Hordeum* species, with a bootstrap value of 50% and ALR of 0.92, suggesting the maternal genome donor of *H. brachyantherum* subsp. *brachyantherum* might come from I genome "new world" diploid *Hordeum* species.

rDNA ITS sequences suggested *H. roshevitzii* as one parent to tetraploid *H. jubatum*, *H. tetraploidum*, and *H. fuegianum* (Blattner, 2004). FISH pattern and RFLP profiles also indicated *H. roshevitzii* as genome donor species to those tetraploid species (Taketa *et al.*, 2005). However, our phylogeny results revealed a much more complicated relationship of the three tetraploids.

Both the DMC1 and HTL phylogenies grouped one copy of *H. tetraploidum* sequences with *H. brevisubulatum* subsp. *violaceum,* strongly supporting that *H. brevisubulatum* might be one possible genome donor of *H. tetraploidum.* In the DMC1 phylogeny, one copy of *H. brachyantherum* subsp. *brachyantherum* is in the same clade as *H. brevisubulatum* subsp. *violaceum* with a high bootstrap value of 94% (Fig. 11). Similarly in HTL phylogeny, one copy of *H. tetraploidum* is in the same clade with both accessions of *H. brevisubulatum* subsp. *violaceum,* with a high bootstrap value of 93% and ALR of 0.98 (Fig. 8). The other HTL copy of *H. tetraploidum* was grouped with *H. patagonicum, H. flexuosum, H. chilense* and *H. pubiflorum* along with polyploids *H. fuegianum, H. lechleri* and *H. parodii,* with a bootstrap value of 63% and ALR of 0.84. This is also supported in the DMC1 phylogeny, where one copy of *H. tetraploidum* was grouped together with *H.*

fuegianum and H. jubatum with a high bootstrap value of 100%, suggesting that *H. tetraploidum* might share a common ancestor with *H. fuegianum.* In the rps16 phylogeny, *H. tetraploidum* was placed in a large clade with mainly I genome "new world" species, instead of *H. brevisubulatum* (Fig. 7). This might suggest that *H. brevisubulatum* is not the maternal genome donor of *H. tetraploidum*, and more research investigating the maternal parent of *H. tetraploidum* is needed.

As for *H. fuegianum*, both the DMC1 phylogeny and HTL phylogeny have grouped one copy of *H. fuegianum* with one copy of *H. tetraploidum.* In the DMC1 phylogeny, the two formed a monophyletic group with *H. jubatum*, with a high bootstrap value of 100%, and *H. brevisubulatum* subsp. *villaceum* was grouped with the other copy of *H. tetraploidum* in a distant clade (Fig. 11). Interestingly, in the HTL phylogeny, one copy of each of *H. fuegianum* and *H. tetraploidum* were in the same clade with both accessions of *H. brevisubulatum* subsp. *violaceum,* with a high bootstrap value of 93% and ALR of 0.98 (Fig. 8). The DMC1 phylogeny only placed the other copy of *H. fuegianum* in a large clade with I genome "new world" diploid species, with a high bootstrap value of 99%. The HTL phylogeny further revealed a close relationship of the other HTL copy of *H. fuegianum* with diploids *H. patagonicum* subsp. *santacruense, H. patagonicum* subsp. *magellanicum, H. flexuosum, H. chilense,* and *H. pubiflorum,* with a bootstrap value of 63% and ALR of 0.84, indicating these diploids might be the other potential genome donor of *H. fuegianum.* The trnD-trnT phylogeny also grouped *H. fuegianum* with *H. pubiflorum* and *H. patagonicum* subsp. *magellanicum,* with ALR of 0.90. This suggests that these two diploids are likely the maternal parent of *H. fuegianum* (Fig. 6). Both the trnD-trnT and rps16 phylogenies

from the chloroplast datasets placed the *H. fuegianum* in a distant clade from *H. brevisubulatum* (Fig. 6, 7). This supports the conclusion that *H. brevisubulatum* might be the paternal parent of *H. fuegianum.* Two distinct copies of rpb2 sequences were also discovered from species *H. fuegianum*. However, due to relatively low variation in *Hordeum* polyploids, we could not infer particular genome donors to *H. fuegianum* (Fig. 9).

Two distinct copies of each of the HTL, DMC1 and RPB2 datasets were discovered for the tetraploid *H. jubatum* (Fig. 8, 9, 11). However, due to the lower level of variation, none of the datasets could identify any particular direct genome donor of *H. jubatum*. RPB2 placed both copies in the large clade together with other I genome "new world" species with a bootstrap value of 76% and ALR of 0.95. The HTL phylogeny further grouped one copy of *H. jubatum* with diploids *H. stenostachys, H. pusillum, H. euclaston, H. erectifilium* and *H. intercedens* as well as one copy each from two other polyploids *H. arizonicum, H. depressum,* with a high bootstrap value of 90% and ALR of 0.91. However, this contradicts with the result from the DMC1 phylogeny, which grouped one copy of *H. jubatum* with one copy each of *H. tetraploidum* and *H. fuegianum,* with a high bootstrap value of 100% and ALR of 1.00. The rps16 phylogeny grouped *H. jubatum* with both accessions of *H. depressum,* with a high bootstrap value of 86% and ALR of 0.98, indicating they might share a common maternal parent. However, the resolution of the rps16 phylogeny is not high enough to infer which particular diploid is the maternal parent. The trnD-trnT phylogeny grouped both accessions of *H. jubatum* with most *Hordeum* diploids distributed in Eurasia, with a high bootstrap value of 100% and ALR of 1.00. This

suggests the maternal genome donor of *H. jubatum* possibly came from the Eurasian continent.

4.3.2. Origin of Hexaploid Species

Among the *Hordeum* hexaploid species, *H. arizonicum* is the only annual/biennial species distributed in North America; the other three are perennials distributed in South America. *H. arizonicum* has been considered to have an allopolyploid origin from a diploid species and a tetraploid species. Rajhathy and Symko (1966) suggested *H. arizonicum* originated from diploid *H. pusillum* and tetraploid *H. jubatum*, which was supported by the rDNA ITS data of Blattner (2004) and FISH and RFLP patterns of Taketa *et al.* (2005). cpDNA analysis suggested that *H. pusillum* could be the maternal parent (Nishikawa *et al*, 2002). Our HTL phylogeny only discovered one copy of HTL sequences from *H. arizonicum,* which was grouped with *H. pusillum* with a bootstrap value of 59% and ALR of 0.79 (Fig. 8). This agrees with previous studies. In addition, our rps16 phylogeny also grouped *H. arizonicum* together with *H. pusillumr*, with a bootstrap value of 64% and ALR of 0.92, suggesting *H. pusillum* as the maternal parent of *H. arizonicum*. The DMC1 phylogeny revealed three DMC1 copies from *H. arizonicum*, suggesting *H. arizonicum* was originated from three distinct genome donors. Phylogenetic analysis revealed a group including *H. arizonicum, H. tetraploidum, H. fuegianum* and *H. jubatum* with 68% support (Fig. 11). The second gene copies of *H. arizonicum* formed a group with polyploid species *H. procerum* and *H. brachyantherum ssp. brachyantherum* with a 63% bootstrap

value, and the third formed a group with polyploid species *H. procerum, H. depressum, H. brachyantherum* subsp. *brachyantherum* and diploid species *H. brachyantherum* subsp. *californicum* with a 92% bootstrap value, sharing a 15 bp deletion (Fig. 10C). According to this sequence alignment and phylogeny tree, *H. brachyantherum* subsp. *californicum* is probably one of the diploid genome donors, and the third genome donor might be shared among *H. procerum, H. brachyantherum* subsp. *brachyantherum* and *H. arizonicum*. It is also possible that *H. arizonicum* did evolve from a diploid parent and an allotetraploid parent, in which case *H. brachyantherum* subsp. *brachyantherum* would be the best candidate, considering it shared a close relationship with both of the other two copies and is distributed in the Southwest of the United States.

Based on C-banding pattern and morphology of marker SAT chromosomes, Linde-Laursen *et al.* (1990) proposed that alloploid *H. parodii* was originated from tetraploid species *H. tetraploidum* and diploid species *H. muticum.* rDNA sequence (Blattner 2004), FISH and RFLP (Taketa *et al.*, 2005) analysis agreed that *H. tetraploidum* is the tetraploid genome donor. In the present study, we only identified two DMC1 copies from *H. parodii* (Fig. 11). Being the only tetraploid species in the monophyletic group with *H. parodii* with 100% bootstrap value, *H. tetraploium* was proposed to be the tetraploid parent, which is consistent with the previous studies. The diploid ancestor suggested in the present study is *H. flexuosum* with 60% bootstrap value. Only one copy of HTL sequences from *H. parodii* was discovered in our study. This copy was grouped with diploids *H. patagonicum* subsp. *santacrucense, H. patagonicum* subsp. *magellanicum, H., flexuosum, H. chilense,* and *H. pubiflorum*

along with polyploids *H. fuegianum, H. lechleri* and *H. tetraploidum,* with a bootstrap value of 63% and ALR of 0.84 (Fig. 8). This also agrees with the DMC1 phylogeny that *H. parodii* has close relationship with *H. tetraploidum* and *H. flexuosum.* In addition, the common distribution of *H. parodii, H. tetraploidum* and *H. flexusum* in South America also supports their close relationship. However, this differs from the results of the rpb2 phylogeny. Two copies of rpb2 sequences from *H. parodii* were discovered (Fig. 9). One copy was grouped together with tetraploid *H. fuegianum,* with a high bootstrap value of 100% and ALR of 1.00. The other copy was placed in the large clade with I genome "new world" diploids, with a bootstrap value of 76% and ALR of 0.95. In the rps16 study, *H. parodii* was grouped with *H. patagonicum* subsp. *mustersii* with a bootstrap value of 61% and ALR of 0.92, indicating *H. patagonicum* subsp. *mustersii* as the possible maternal genome donor (Fig. 7).

H. lechleri, a South American hexaploid species, was suggested to have the tetraploid species *H. jubatum* as one of the genome donors (Taketa *et al.*, 2005). However, in our study there was only one DMC1 gene copy identified from *H. lechleri,* which formed a monophyletic group together with polyploidy *Hordeum* species *H. tetraploidum, H. parodii* and *H. procerum* with 100% bootstrap value (Fig. 11), suggesting these four species share a common ancestor for one genome. This is partly consistent with the HTL phylogeny. Two distinct copies of HTL sequences from *H. lechleri* were discovered in the present study (Fig. 8). Similarly, one copy of *H. lechleri* was also grouped with one copy of *H. tetraploidum, H. procerum* and *H. brachyantherum* subsp. *brachyantherum,* with a high bootstrap value of 88% and

ALR of 0.92. Furthermore, this clade was grouped with both accessions of *H. brevisubulatum* subsp. *violaceum*, indicating that *H. brevisubulatum* subsp. *violaceum* could be the possible genome donor of *H. lechleri*. The other HTL copy of *H. lechleri* was grouped with diploids *H. patagonicum* subsp. *santacrucense, H. patagonicum* subsp. *magellanicum, H., flexuosum, H. chilense,* and *H. pubiflorum* along with polyploids *H. fuegianum, H. parodii* and *H. tetraploidum,* with a bootstrap value of 63% and ALR of 0.84. However, we could not identify the other direct genome donor to *H. lechleri* from the HTL phylogeny. As for the rpb2 phylogeny, only one copy was discovered for *H. lechleri*, which was grouped in the large clade with I genome "new world" diploids and some other polyploids with a bootstrap value of 76% and ALR of 0.95 (Fig. 9). Due to low resolution, we could not identify the direct genome donor to *H. lechleri* from the rpb2 phylogeny either. The rps16 phylogeny also placed *H. lechleri* in the large clade with I genome "new world" diploids, with a bootstrap value of 50% and ALR of 0.92.

H. procerum has been considered as an allopolyploid species. C-banding pattern and morphology of SAT chromosomes suggested *H. cordobense* as one donor genome to *H. procerum* (Linde-Laursen *et al.* 1990). Blattner (2004) proposed diploid species *H. cordobense* and tetraploid species *H. tetraploidum* as the parents of this hexaploid. Taketa *et al.* (2005) also suggested the possibility of *H. tetraploidum* being one of the ancestors. Unexpectedly, in the present study we identified four DMC1 gene copies from hexaploid species *H. procerum* (Fig. 11). One copy was grouped with diploid species *H. cordobense* with 86% bootstrap value in agreement with previous studies

that *H. cordobense* is one of its diploid genome donors (Linde-Laursen *et al.* 1990; Blattner 2004). The sequence alignment in our study also revealed that the two species exclusively shared a 24 bp direct repeat (Fig. 10B). Another copy was grouped with polyploid species *H. tetraploidum, H. lechleri,* and *H. parodii* with 100% bootstrap value, suggesting their common origin, which supported the suggestions of Blattner (2004) and Taketa *et al.* (2005). Of the other two copies, one formed a monophyletic group with polyploid species *H. procerum, H. brachyantherum* subsp. *brachyantherum* and *H. arizonicum* with 63% bootstrap value, and the other formed a monophyletic group with polyploid species *H. arizonicum, H. depressum, H. brachyantherum* subsp. *brachyantherum* and diploid species *H. brachyantherum* subsp. *californicum* with 92% bootstrap value (sharing a 15 bp deletion). The fact that more than three DMC1 gene copies were discovered from this hexaploid species may be explained by gene introgression, since this phenomenon was previously reported in Triticeae genus *Elymus* (Mason-Gamer, 2004; Fortune *et al.*, 2008). In the HTL study, three distinct copies of HTL sequences from *H. procerum* were discovered (Fig. 8). One copy was grouped with *H. tetraploidum, H. brachyantherum* subsp. *brachyantherum* and *H. lechleri* with a high bootstrap value of 88% and ALR of 0.92. This clade was then grouped with both accessions of *H. brevisubulatum* subsp. *violaceum,* with a high bootstrap value of 93% and ALR of 0.98, indicating their common ancestor diploid *H. brevisubulatum* subsp. *violaceum.* This is also consistent with the DMC1 phylogeny that one copy of each of *H. procerum, H. tetraploidum, H. parodii,* and *H. lechleri* was grouped with *H. brevisubulatum* subsp. *violaceum,* with a high bootstrap value of 94%. This is further

supported by the rpb2 phylogeny. Two distinct copies of rpb2 sequences from *H. procerum* were discovered (Fig. 9). One copy was grouped with both accessions of *H. brevisubulatum* subsp. *violaceum* with a bootstrap value of 70%. The other copy was grouped with *H. parodii* with a bootstrap value of 69% and ALR of 0.80. In addition, both rps16 and trnD-trnT phylogenies separated *H. procerum* from *H. brevisubulatum* subsp. *violaceum,* suggesting *H. brevisubulatum* subsp. *violaceum* is likely the paternal parent of *H. procerum* (Fig.6, 7) However, due to low resolution, both rps16 and trnD-trnT phylogenies could not identify the direct maternal donor of *H. procerum.*

5. Conclusion

The present study further concluded that incomplete lineage sorting is the major cause for the discrepancy of the positons of *H. marinum* subspecies. The incongruence of the positions of the three Eurasian I genome species *H. roshevizii, H. bodgnai,* and *H. brevisubulatum* between nuclear datasets and chloroplast datasets could be explained that these three species share a common distribution with other genome group species in Eurasia areas and therefore their chloroplast sequences remain more similar, while these three species are evolutionarily more related to American species of the same genome group I and thus they are grouped together with all other I genome species in the nuclear gene phylogeny .

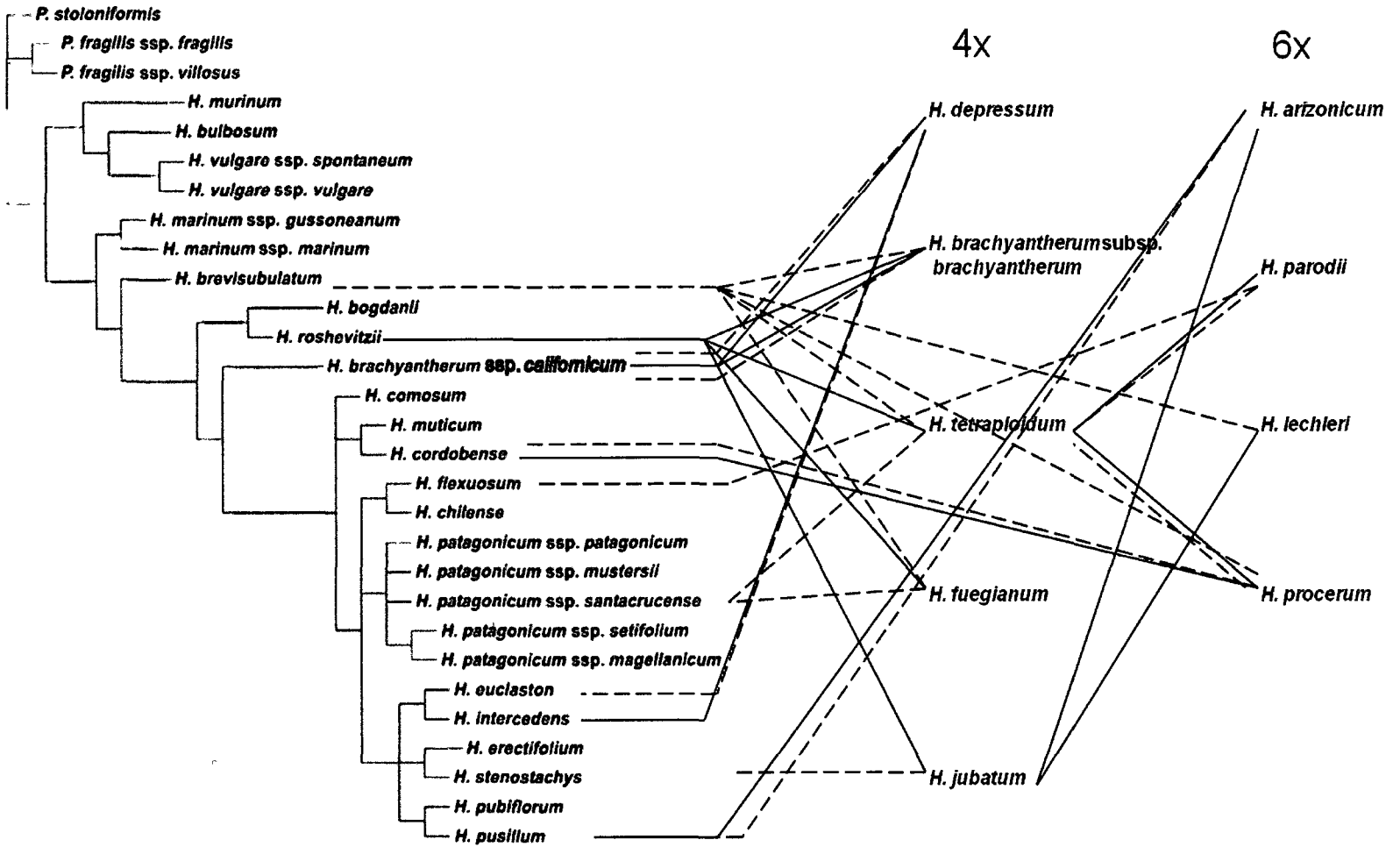
Our study also revealed origins of *Hordeum* polyploid species (Fig. 12). For tetraploid species, previous studies suggesting *H. brachyantherum* subsp.

californicum and *H. intercedens* are the parents of *H. depressum*. While our study supports *H. brachyantherum* subsp. *californicum* as the parent, it also suggests that *H. euclaston* could be the other possible genome donor. Our study also further confirms that *H. brachyantherum* subsp. *californicum* is one genome donor of *H. brachyantherum* subsp. *brachyantherum.* However, our study does not favor *H. roshevitzii* as the other parent as suggested in previous studies, but suggests that *H. brevisubulatum* is likely the other parent. *Hordeum roshevitzii* was regarded as the possible genome donor for *H. tetraploidum* and *H. fuegianum.* Our study revealed a closer relationship of the *H. tetraploidum* and *H. fuegianum* with *H. brevisubulatum*, suggesting *H. brevisubulatum* could be the possible genome donor. The other genome donor of the two tetraploids might come from *H. patagonicum* subspecies. Our study does not favor the conclusion that *H. roshevitzii* is one parent for *H. jubatum*..

As for the hexaploid species, our study further confirms that *H. pusillum* is the maternal parent of hexaploid species *H. arizonicum,* and supports that *H. tetraploidum* is the possible tetraploid genome donor of *H. parodii* and *H. procerum.* We also confirm *H. cordobense* as the diploid genome donor of *H. procerum.* In addition, our study suggests *H. flexuosum* might the diploid genome donor of *H. parodii.* Previous suggestion on *H. jubatum* as the tetraploid genome donor to *H. lechleri* is not supported by our study. However, we did reveal that *H.brevisubulatum* is the possible diploid genome parent of *H. lechleri.*

Figure 12

The origins of *Hordeum* polyploidy species. Solid lines refer to the results of polyploid origins in previous studies. Dash lines refer to the result from the present study of polyploidy origins.


P. stoloniformis
P. fragilis ssp. fragilis
P. fragilis ssp. villosus
H. murinum
H. bulbosum
H. vulgare ssp. spontaneum
H. vulgare ssp. vulgare
H. marinum ssp. gussoneanum
H. marinum ssp. marinum
H. brevisubulatum
H. bogdanii
H. roshevitzii
H. brachyantherum ssp. californicum
H. comosum
H. muticum
H. cordobense
H. flexuosum
H. chilense
H. patagonicum ssp. patagonicum
H. patagonicum ssp. mustersii
H. patagonicum ssp. santacrucense
H. patagonicum ssp. setifolium
H. patagonicum ssp. magellanicum
H. euclaston
H. intercedens
H. erectifolium
H. stenostachys
H. pubiflorum
H. pusillum
4x
6x
H. depressum
H. brachyantherum subsp. brachyantherum
H. tetraploidum
H. fuegianum
H. jubatum
H. arizonicum
H. parodii
H. lechleri
H. procerum

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