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ARTICLE

Phylogenetic analysis of two single-copy nuclear genes revealed origin and complex relationships of polyploid species of *Hordeum* in Triticeae (Poaceae)

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Abstract: Two single-copy nuclear genes, the second largest subunit of RNA polymerase II (*RPB2*) and thioredoxin-like gene (*HTL*), were used to explore the phylogeny and origin of polyploid species in *Hordeum*. Our results were partly in accord with previous studies, but disclosed additional complexity. Both *RPB2* and *HTL* trees confirmed the presence of **Xa** genome in *H. capense* and *H. secalinum*, and that *H. depressum* originated from *H. californicum* together with other American diploids, either *H. intercedens* or *H. pusillum*. American diploids solely contributed to the origin of *H. depressum*. The Asian diploids, either *H. bogdanii* or *H. brevisubulatum*, contributed to the formation of American polyploids except *H. depressum*. *RPB2* and *HTL* sequences showed that *H. roshevitzii* did not contribute to the origin of American tetraploids. Our data showed a close relationship between the hexaploids *H. procerum* and *H. parodii* and the tetraploids *H. brachyantherum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum*, and *H. tetraploidum*. The involvement of the diploid *H. pusillum* and the tetraploid *H. jubatum* in the formation of *H. arizonicum* was also indicated in the *HTL* phylogeny. Our results suggested a possible gene introgression of **W**- and **P**-genome species into the tetraploid *H. jubatum* and the hexaploid *H. procerum*.

Key words: Hordeum, Triticeae, polyploidization, phylogeny, nuclear gene.

Résumé : Deux gènes nucléaires à simple copie, la deuxième plus grande sous-unité de l'ARN polymérase II (*RPB2*) et un gène de type thioredoxine (*HTL*), ont été employés pour explorer la phylogénie et l'origine des espèces polyploïdes au sein du genre *Hordeum*. Les résultats obtenus sont en accord partiel avec les études antérieures, mais ont révélé une complexité additionnelle. Les deux arbres obtenus (avec *RPB2* et *HTL*) ont confirmé la présence du génome **Xa** chez le *H. capense* et le *H. secalinum*, de même que le *H. depressum* originerait du *H. californicum* avec des apports d'autres diploïdes américains, soit le *H. intercedens* ou le *H. pusillum*. Les diploïdes américains seraient les seuls à avoir contribué au *H. depressum*. Les diploïdes asiatiques, soit le *H. bogdanii* ou le *H. brevisubulatum*, auraient contribué à la formation des polyploïdes américains à l'exception du *H. depressum*. Les séquences de *RPB2* et *HTL* ont montré que le *H. roshevitzii* n'a pas contribué à l'origine des tétraploïdes américains. Ces données montrent une relation proche entre les hexaploïdes *H. procerum* et *H. parodii* et les tétraploïdes *H. brachyantherum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum* et *H. tetraploidum*. L'implication du diploïde *H. pusillum* et du tétraploïde *H. jubatum* dans la formation du *H. arizonicum* a aussi été suggérée par la phylogénie *HTL*. Ces résultats suggèrent une possible introgression génique, à partir des espèces à génomes **W** et **P**, chez le tétraploïde *H. jubatum* et l'hexaploïde *H. procerum*. [Traduit par la Rédaction]

Mots-clés : Hordeum, Triticeae, polyploïdisation, phylogénie, gène nucléaire.

Introduction

The genus *Hordeum* in Triticeae includes 31 species (16 diploids, 11 polyploids, and 4 with both diploid and polyploidy types) with a basic chromosome number of $x = 7$. These species are distributed disjunctively in Asia, America, and South Africa (von Bothmer et al. 1995). Based on chromosome banding information, the diploid species have been divided into four monogenomic groups (see Blattner 2009 for genome designation): the **H**-genome group (*H. vulgare* and *H. bulbosum*), the **Xa**-genome group (*H. marinum*), the **Xu**-genome group (*H. murinum*), and the **I**-genome group (the remaining diploid species) (von Bothmer et al. 1986). The presence of four basic genomes is also supported by isoenzyme data (Jørgensen 1986), C-banding data (Linde-Laursen et al. 1992), and restriction fragment length polymorphism with repetitive DNA (Svitashev

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et al. 1994), nuclear gene *DMC1* (disrupted meiotic cDNA1) sequences (Petersen and Seberg 2003), and rDNA ITS sequence (Blattner 2004).

Polyploids occur in all the four genomic groups in the genus *Hordeum*. All polyploid species are alloploids or at least segmental alloploids, except for *H. bulbosum* and *H. brevisubulatum* which are of autopolyploid origin (von Bothmer and Komatsuda 2011). Fluorescence in situ hybridization (FISH) and rDNA-RFLP patterns suggest that the Asian *H. roshevitzii* and American *H. californicum* are progenitors of the tetraploid species *H. brachyantherum*, *H. fuegianum*, *H. jubatum*, and *H. tetraploidum* (Taketa et al. 2005). The study of Blattner (2006) indicated that the American *H. californicum* and the Asian *H. roshevitzii* were ancestors to *H. jubatum*.

Single copy or low copy nuclear genes are bi-parentally inherited, and less likely to be subject to concerted evolution, and thus they are ideal for phylogenetic studies (Small et al. 2004; Sun et al. 2009). They have been widely used to construct phylogenetic trees and to investigate the polyploid origins of plants (Petersen and Seberg 2000, 2004; Blattner 2006; Sun et al. 2007, 2008, 2009). A few such genes have been used to infer relationship in polyploid species of *Hordeum* (Wang and Sun 2011; Brassac et al. 2012).

Low-copy nuclear gene *TOPO6* data suggest that three species are involved in the evolution of American polyploids, and all hexaploid species from the New World. They all have a copy from the American *H. californicum* or an extinct species closely related to *H. intercedens* (Komatsuda et al. 2009; Brassac et al. 2012; Brassac and Blattner 2015). The studies mentioned above enhance our understanding of the origin and evolutionary history of the genus, but the origin and phylogeny of polyploid species of *Hordeum* remain unclear.

The aim of the present study was to elucidate the origin of polyploid species of *Hordeum* and to explore phylogenetic relationships between these polyploids and the diploid species in this genus and in the tribe Triticeae using two single-copy nuclear regions, the second largest subunit of RNA polymerase II (*RPB2*) and thioredoxin-like gene (*HTL*).

Materials and methods

Plant material

Thirty-four accessions of 15 polyploid species/subspecies of *Hordeum* were included in the study. The seeds were kindly provided by the Nordic Genetic Resource Center, Sweden. Collector or donor for each polyploid species of *Hordeum* used in this study can be found at the Nordic Genetic Resource Center website (http://www.nordgen.org/index.php/en/content/view/full/344). *Bromus catharticus* was provided by the Plant Gene Resource of Canada. Germinated seeds were transplanted into a sand–peat mixture, and the plants were maintained in a greenhouse. DNA was extracted from young freeze-dried tissue using the method of Junghans and Metzlaff (1990). Voucher specimens from some accessions were made of the mature plants and used for checking the identification of the seed and deposited at the Biology Department, Saint Mary's University. Plant materials with accession number, genome constitution, country of origin, and gene sequence availability are presented in Table 1. The sequences of diploid species from genera *Aegilops*, *Agropyron*, *Australopyrum*, *Dasypyrum*, *Heteranthelium*, *Psathyrostachys*, *Pseudoroegneria*, and *Thinopyrum* used in this study were downloaded from the GenBank. The number of sequences from each species is given in Table 1.

DNA amplification and sequencing

The second largest subunit of RNA polymerase II (*RPB2*) and the thioredoxin-like gene (*HTL*) sequences were amplified by polymerase chain reaction (PCR) using the primers P6F/P6FR (Sun et al. 2007) and trxF/R (Kakeda et al. 2008), respectively. Amplification of DNA was carried out in a 20 μL reaction mixture containing 30 ng template DNA, 0.2 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 1 U of high fidelity *Taq* DNA polymerase (Biolabs, New England), and distilled deionized water to the final volume. The mixture was amplified using the Bio-Rad icycler® thermal cycler. The amplification profile for the *RPB2* gene was as follows: an initial denaturation at 95 °C for 4 min; 35–40 cycles of 95 °C for 40 s, 52 °C for 40 s, 72 °C for 90 s; and a final cycle of 72 °C for 10 min. The amplification profile for the *HTL* gene was as follows: an initial denaturation at 95 °C for 4 min; 14 cycles of 95 °C for 40 s denaturing and 72 °C for 90 s extension. Annealing (40 s) temperatures were progressively decreased by 0.5 °C every cycle from 57 to 50 °C. The PCR reaction continued for 29 additional cycles at 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 90 s. The reaction ended with a 10 min extension at 72 °C.

To avoid any error that would be induced by *Taq* DNA polymerase during PCR amplification, each PCR product was independently amplified twice, and cloned into pGEM-easy T vector (Promega Corporation, Madison, Wis., USA) according to the manufacturer's instruction. Plasmid DNA was isolated using Promega Wizard® Plus Minipreps DNA Purification System (Promega Corporation, Madison, Wis., USA) according to the manufacturer's instructions. Plasmids were commercially sequenced by Taihe Biotechnology (Beijing, China). At least 10 clones from the PCR product of each accession were sequenced.

Data analysis

Automated sequence outputs were visually inspected with chromatographs. Multiple sequence alignments were made using ClustalX using default parameters. Multiple sequences from each accession were first compared, and only one copy of the sequence was kept from the identical copies. The remaining sequences from each accession were phylogenetically analyzed to figure out

Table 1. Taxa from *Aegilops*, *Hordeum*, *Pseudoroegneria*, *Psathyrostachys*, *Thinopyron*, *Agropyron*, *Australopyrum*, *Heteranthelium*, and *Dasypyrum* used in this study.

Species	Taxa accession No.	Genome	Origin	*RPB2**	*HTL**
Aegilops umbellulata Zhuk.	PI 542378	**U**	Turkey	–	+
Aegilops uniaristata Vis.	PI 554418	**N**	Former Soviet Union	–	+
Agropyron cristatum (L.) Gaertn.	PI 383534	**P**	Kars, Turkey	EU187438	–
Australopyrum retrofractum (Vickery) Á. Löve	PI 533014	**W**	New South Wales, Australia	+	+
Bromus catharticus Vahl	CN32048		Unknown	HQ014410	–
Dasypyrum villosum (L.) P. Candargy	PI 368886	**V**	Gaziemir, Turkey	EU187471	–
Heteranthelium piliferum (Banks & Sol.) Hochst.	PI 401351	**Q**	Iran	–	+
Hordeum bogdanii Wilensky	H4014	**I**	Pakistan	–	+
	H4014	**I**	Pakistan	+	–
Hordeum brachyantherum Nevski subsp. *californicum*	H3317	**I**	USA	+	–
(Covas and Stebbins) Bothm. et al.	H3317	**I**	USA	–	+
	H1954	**I**	USA	–	+
Hordeum brevisubulatum (Trin.) Link	H316	**I**	Iran	–	+
	H304	**I**	Turkey	–	+
Hordeum bulbosum L.	710–17	**H**	Morocco	–	+
	H3878	**H**	Italy	+	–
Hordeum chilense Roem. and Schult.	Camb.line1	**I**	Unknown	–	+
	H1816	**I**	Chile	+	–
Hordeum comosum Presl.	H1333	**I**	Argentina	+	–
	H10608	**I**	Argentina	–	+
Hordeum cordobense Bothmer, Jacobsen and Nicora	H6460	**I**	Argentina	+	–
Hordeum erectifolium Bothmer, Jacobsen and Jørg.	H1150	**I**	Argentina	+	+
Hordeum euclaston Steud.	H2148	**I**	Uruguay	+	–
	H1103	**I**	Argentina	N/A	+
Hordeum flexuosum Steud.	H2127	**I**	Uruguay	+	–
	H1112	**I**	Argentina	N/A	+
Hordeum intercedens Nevski	H1941	**I**	USA	+	–
	H2310	**I**	USA	N/A	+
Hordeum marinum Huds. subsp. *gussoneanum* (Parl.)	H539	**Xa**	Spain	–	+
Thell.	H28	**Xa**	Hungary	–	+
	H155	**Xa**	Greece	+	–
Hordeum marinum Huds. subsp. *marinum*	H515	**Xa**	Spain	–	+
	H41	**Xa**	Turkey	–	+
	H121	**Xa**	Greece	+	–
Hordeum murinum L. subsp. *glaucum* (Steud.) Tzvel.	JIC line 71	**Xu**	Unknown	–	+
	H10289	**Xu**	Tajikistan	–	+
	H74	**Xu**	Egypt	+	–
	H52	**Xu**	Jordan	+	–
Hordeum muticum J. Presl.	H6479	**I**	Argentina	+	–
	H6470	**I**	Argentina	N/A	+
Hordeum patagonicum (Hauman) Covas subsp.	H1342	**I**	Argentina	+	–
magellanicum (Parodi and Nicora) Bothm. et al.	H1368	**I**	Chile	N/A	+
Hordeum patagonicum (Hauman) Covas subsp. *patagonicum*	H6052	**I**	Argentina	+	–
Hordeum patagonicum (Hauman) Covas subsp. *santacrucense* (Parodi and Nicora) Bothm. et al.	H1353	**I**	Argentina	+	–
Hordeum patagonicum (Hauman) Covas subsp. *setifolium* (Parodi and Nicora) Bothm. et al.	H1352	**I**	Argentina	+	–
Hordeum patagonicum (Hauman) Covas subsp. *mustersii* (Nicora) Bothm. et al.	H1358	**I**	Argentina	+	N/A
Hordeum pubiflorum Hook. f	H1296	**I**	Argentina	AY137402	+
	H1236	**I**	Argentina	+	–
Hordeum pusillum Nutt.	H2038	**I**	New Mexico, USA	–	+
	H2024	**I**	USA	+	–
Hordeum roshevitzii Bowden	H9152	**I**	China	+	+

Table 1 (*concluded*).

Species	Taxa accession No.	Genome	Origin	*RPB2**	*HTL**
Hordeum stenostachys Godr.	H1780	**I**	Argentina	+	–
	H6439	**I**	Argentina	N/A	+
Hordeum vulgare subsp. *spontaneous* (K. Koch) Thell.	H3140A	**H**	Cyprus	+	–
Hordeum vulgare subsp. *spontaneum*	OUH620	**H**	Morocco	–	+
Hordeum vulgare subsp. *vulgare*	Bonus	**H**	Sweden	–	+
	H7514A	**H**	China	+	–
Hordeum arizonicum Covas	H3255	**III**	USA	+,+,+ (10)	+,+,+,+ (10)
	H3253	**III**	USA	–	+,+,+ (10)
	H2313	**III**	USA	–	+,+,+ (10)
Hordeum brachyantherum Nevski subsp. *brachyantherum*	H10227	**II**	Tadzhikistan	+,+ (10)	–
	H1952	**II**	USA	–	+,+ (10)
	H2318	**II**	USA	+,+ (10)	+,+ (10)
Hordeum capense Thunb.	H335	**IXa**	South Africa	+,+ (13)	+,+ (10)
	H3923	**IXa**	South Africa	–	+,+ (10)
Hordeum depressum (Scribn. & J.G. Sm.) Rydb.	H2005	**II**	USA	+,+ (10)	+,+ (10)
	H723	**II**	USA	+,+ (10)	+,+ (10)
	H2008	**II**	USA	N/A	+,+ (10)
	H2089	**II**	USA	+	N/A
Hordeum fuegianum Bothmer, Jacobsen & Jørg.	H1371	**II**	Argentina	+,+ (10)	+,+ (10)
	H1422	**II**	Argentina	+,+ (10)	+,+ (10)
	H6168	**II**	Argentina	+,+ (10)	N/A
Hordeum guatemalense Bothmer et al.	H2299	**II**	Mecixo	+,+ (10)	+,+ (10)
Hordeum jubatum L.	H1935	**II**	Mexico	+,+ (10)	+,+ (10)
	H1162	**II**	Mexico	+,+ (10)	+,+ (10)
Hordeum lechleri (Steud.) Schenck	H1437	**III**	Argentina	+ (10)	+,+,+ (10)
	H6344	**III**	Argentina	N/A	+,+ (10)
Hordeum marinum subsp. *gussoneanum*	H2303	**XaXa**	USA	–	+,+ (10)
	H819	**XaXa**	Turkey	–	+,+ (10)
Hordeum murinum subsp. *leporinum*	H509	**XuXu**	Spain	–	+,+ (10)
Hordeum murinum subsp. *murinum*	H614	**XuXu**	Greece	–	+,+ (10)
Hordeum parodii Covas	H1458	**III**	Argentina	+,+,+ (10)	+,+,+ (10)
	H1444	**III**	Argentina	+,+,+ (10)	+,+,+ (10)
	H1146	**III**	Argentina	+,+ (10)	–
Hordeum procerum Nevski	H1781	**III**	Argentina	+,+,+ (10)	+,+ (10)
	H1166	**III**	Argentina	–	+,+ (10)
	H1137	**III**	Argentina	+,+,+ (10)	+,+,+ (10)
	H1156	**III**	Argentina	+,+	–
Hordeum secalinum Schreb.	H231	**IXa**	Sweden	+,+ (10)	+ (10)
Hordeum tetraploidum Covas	H1466	**II**	Argentina	+,+ (11)	+,+ (10)
	H1489	**II**	Argentina	+,+ (10)	+,+ (10)
Pseudoroegneria libanotica (Hack.) D.R. Dewey	PI 228389	**St**	Iran	HQ231837	+
Pseudoroegneria libanotica (Hack.) D.R. Dewey	PI 330688	**St**	Sirak—Sar, Iran	EF596751	–
	PI 401274	**St**	Saqqez, Iran	EF596752	–
Psathyrostachys juncea	H10108	**Ns**	Russian	–	+
Pseudoroegneria spicata (Pursh) Á. Löve	PI 232134	**St**	Wyoming, United States	N/A	HQ231841
	PI 506274	**St**	Washington, United States	EF596746	–
	PI 610986	**St**	Utah, United States	EF596747	–
Pseudoroegneria *stipifolia* (Czern. ex Nevski) Á. Löve	PI 325181	**St**	Stavropol, Russian Federation	EF596748	+
Pseudoroegneria *strigosa* subsp. *aegilopoides*	PI 440000	**St**	Stavro, Russian Federation	HQ231847	+
Thinopyrum bessarabicum (Savul. & Rayss) Á. Löve	PI 531712	**E**^{b}	Estonia	EU187474	–
Thinopyrum elongatum (Host) D.R. Dewey	PI 142012	**E**^{e}	Odessa, Russian Federation	EU187439	–

Note: The *RPB2* and *HTL* sequences from polyploid species of *Hordeum* were sequenced and analyzed.

*The plus sign (+) indicates that sequence data has been recovered; the minus sign (–) indicates no sequence data has been recovered; the number in parenthesis indicates the number of colonies sequenced.

distinct copies. Alignments were inspected for chimeric sequences caused by PCR amplification, RDP4 was used to detect and analyze recombination (Martin et al. 2015), and no recombinants were detected. Phylogenetic analysis using the maximum-parsimony (MP) method was performed with the computer program PAUP* ver. 4 beta 10 (Swofford 2003). All characters were specified as unweighted and unordered. The most parsimonious trees were constructed by performing a heuristic search using Tree Bisection-Reconnection (TBR) with the following parameters: MulTrees on and 10 replications of random addition sequences with the stepwise addition option. A strict consensus tree was generated from multiple parsimonious trees. The consistency index (CI) and the retention index (RI) were used to estimate the overall character congruence. Bootstrap (BS) values with 1000 replications (Felsenstein 1985), whaich was calculated by performing a heuristic search using the TBR option with Multree on, were used to test the robustness of clades.

In addition to MP analysis, Bayesian analyses were performed. The best-fitting model of sequence evolution were tested for each data set using jModelTest 2.1.10 (Darriba et al. 2012), using default parameters. The Akaike information criterion (AIC) (Akaike 1973), Bayesian information criterion (BIC) (Schwarz 1978), and a decision-theoretic performance-based approach (DT) (Minin et al. 2003) were estimated for each data. The BIC was used for model selection since it has high accuracy (Darriba et al. 2012). The HKY+G and K80+G substitution models led to the best BIC scores for *RPB2* and *HTL*, respectively. Therefore, the HKY+G (for *RPB2*) and K80+G (for *HTL*) models were used in the Bayesian analysis using MrBayes 3.1 (Ronquist and Huelsenbeck 2005). MrBayes 3.1 was run with the program's standard setting of two analyses in parallel, each with four chains, and estimates of convergence of results were determined by calculating standard deviation of split frequencies between analyses. In total, 1 548 000 generations for *RPB2* and 1 157 000 generations for *HTL* were run to make the standard deviation of split frequencies <0.01. Convergence of the runs was assessed using TRACER 1.5 (Rambaut and Drummond 2007). Samples were taken every 1000 generations. For all analyses, the first 25% of samples from each run were discarded as burn-in to ensure the stationary of the chains. Bayesian posterior probability (PP) values, which were obtained from a majority rule consensus tree generated from the remaining sampled trees, were used to test the robustness of clades.

Results

RPB2 sequence analysis

The DNA from 23 accessions of 12 polyploid species of *Hordeum* were amplified and cloned. Forty-seven *RPB2* sequences were obtained. Two and three distinct copies of sequence from each tetraploid and hexapod species was recovered, respectively, for most polyploids. However, we only successfully obtained one copy of *RPB2* sequence from hexaploid *H. lechleri* (H1437), one copy from accession H2089 of tetraploid *H. depressum*, and two distinct copies from accession H1156 of hexaploid *H. procerum* (Table 1).

The 47 *RPB2* sequences from polyploid species of *Hordeum* were phylogenetically analyzed together with 38 *RPB2* sequences from diploid species of *Hordeum* and other diploid species in the tribe Triticeae. *Bromus catharticus* was used as the outgroup. The data matrix contained 741 characters, of which 419 were constant, 102 were parsimony uninformative, and 220 were parsimony informative. Maximum parsimony analysis produced 596 equally parsimonious trees with CI = 0.743 and RI = 0.891 (excluding uninformative characters). The separated Bayesian analyses using the HKY+G model resulted in identical trees with mean log-likelihood values of −4746.93 and −4744.39. The tree topologies in Bayesian trees are similar to those generated by MP with minor differences. The consensus tree generated from Bayesian analysis with PP value is shown in Fig. 1.

Phylogenetic analyses based on *RPB2* sequence data grouped almost all sequences from species of *Hordeum* into a clade with 0.94 PP support, except one sequence each from accessions H1137 of *H. procerum* and H1162 of *H. jubatum*, and the sequences from the **Xu**-genome species *H. murinum* subsp. *glaucum* (Fig. 1). The **Xu**-genome species *H. murinum* subsp. *glaucum* is sister to **St** (*Pseudoroegneria*) and **V** (*Dasypyrum*) genome species with 0.98 PP support. One sequence each from accessions H1137 of *H. procerum* and H1162 of *H. jubatum* formed a group with *Australopyrum retrofractum* (**W**) and *Agropyron cristatum* (**P**) with 0.88 PP support. Within the *Hordeum* clade, the sequences from diploids and polyploids were divided into several subclades. Twelve sequences from diploid species and 26 sequences from 10 polyploids formed subclade I with 0.99 PP support, in which tetraploid *H. depressum* (1 of 2 copies) and diploid *H. intercedens* (PP = 0.92) were grouped together, and tetraploid *H. depressum* (1 of 2 copies) and diploid *H. californicum* formed a group (PP = 0.86). Hexaploid *H. parodii* and tetraploid *H. tetraploidum* were placed together with diploid *H. chilense* (PP = 0.98). Subclade II is comprised of sequences from diploid *H. bogdanii* and 13 sequences from 8 polyploids (PP = 0.92). The sequences from tetraploids *H. secalinum* and *H. capense*, and diploid *H. marinum* formed subclade III with PP = 1.00 support. A well-supported subclade IV (PP = 0.99) consisted of the sequences from diploid *H. patagonicum* and one sequence each from tetraploids *H. secalinum*, *H. capense*, and *H. fuegianum*, and hexaploid *H. procerum*. The **H**-genome species were found exclusively in one subclade (PP = 0.99).

HTL sequence analysis

A total of 64 *HTL* sequences from 29 accessions of 15 polyploid species of *Hordeum* were obtained. As expected, two and three distinct copies of sequence for each tetraploid and hexaploid species were successfully

Fig. 1. Consensus tree generated from *RPB2* sequence data using Bayesian analysis. Numbers above branches are Bayesian posterior probability (PP) values. *Bromus catharticus* was used as the outgroup. The number preceding the species name is the taxa accession number.

identified, respectively. However, only one copy of *HTL* sequence from tetraploid *H. secalinum* (H231), two distinct copies each from accessions H1781 and H1166 of hexaploid *H. procerum*, and four copies from accession H3255 of hexaploid *H. arizonicum* were recovered (Table 1).

A total of 100 *HTL* sequences including 65 from polyploid species of *Hordeum*, 26 from diploid species of *Hordeum* and, 9 from other diploid species in Triticeae were phylogenetically analyzed using *Psathyrostachys juncea* as the outgroup. The data matrix contained 1184 characters, of which 632 were constant, 139 were parsimony uninformative, and 413 were parsimony informative. Maximum parsimony analysis produced 832 equally parsimonious trees with CI = 0.804 and RI = 0.928 (excluding uninformative characters). The separated Bayesian analyses using the K80+G model resulted in identical trees with arithmetic mean log-likelihood values of –7176.31 and –7180.90. All analyses algorithms resulted in very similar tree topologies with minor differences between the Bayesian tree and those generated by MP. The consensus tree generated from Bayesian analysis with PP value is shown in Fig. 2. All analyses revealed the sequences from species of *Hordeum* to be monophyletic with 0.99 PP support. Four major clades exist in *Hordeum*, concurring with the four assigned genomes (**H**, **I**, **Xa**, and **Xu**). Sister relationships of **H**/**Xu** and **I**/**Xa** taxa were obvious.

In the **Xu**-genome clade, two distinct copies of sequence from tetraploids *H. murinum* subsp. *leporinum* (H509) and *H. murinum* subsp. *murinum* (H614) were clustered together with diploid *H. murinum* (JIC) in one strongly supported clade (1.00 PP). One clade consisting of polyploid species was sister to a clade containing di- and polyploid individuals (Fig. 2).

The **Xa**-genome sequences formed two strongly supported groups (Fig. 2): one sequence each from accessions H2303 and H819 of tetraploid *H. marinum* subsp. *gussoneanum* and sequences from diploid *H. marinum* subsp. *marinum* (H515 and H41) in one group (0.99 PP); one sequence each from accessions H2303 and H819 of tetraploid *H. marinum* subsp. *gussoneanum*, sequences from diploid *H. marinum* subsp. *gussoneanum* (H28 and H539), two sequences from accessions H335 and H3923 of tetraploid *H. capense*, and one sequence from tetraploid *H. secalinum* (H231) in the second group.

The sequences from **I**-genome species were clustered in the **I**-genome group, except the sequences from tetraploid *H. capense* containing **IXa** genomes (Fig. 2). Two copies of sequence from *H. capense* were placed into the **Xa** group. In the **I**-genome group, a clade (0.99 PP) consisting of sequences from diploids *H. roshevitzii* and *H. bogdanii* was sister to a large clade with bichotomy. Subclade Ia (1.00 PP) consisted of sequences from the tetraploids *H. brachyantherum*, *H. fuegianum*, *H. jubatum*, and *H. tetraploidum*, hexaploids *H. arizonicum*, *H. lechleri*, *H. parodii*, and *H. procerum*, and diploid *H. brevisubulatum*. Subclade Ib (0.99 PP) consisted of sequences from the diploids *H. californicum*, *H. chilense*, *H. comosum*, *H. erectifolium*, *H. euclaston*, *H. flexuosum*, *H. intercedens*, *H. patagonicum* subsp. *magellanicum*, *H. muticum*, *H. pubiflorum*, *H. pusillum*, and *H. stenostachys*, tetraploids *H. brachyantherum*, *H. depressum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum*, and *H. tetraploidum*, and hexaploids *H. arizonicum*, *H. lechleri*, *H. parodii*, and *H. procerum*. In addition, most polyploid-derived sequences in this subclade were grouped together with specific diploids into groups (Fig. 2). The distinct copies of sequence from each polyploid were separated into the Ia and Ib subclades, except those from tetraploids *H. depressum* (H2005, H2008, H723) and *H. guatemalense* (H2299) that were placed in the Ib subclade, and sequences from accession H1422 of *H. fuegianum* that were placed in the Ia subclade. In addition, two copies of sequence from accession H1162 of *H. jubatum* were clustered into the Ia subclade, while the sequences from accession H1935 of *H. jubatum* were clustered into the Ib subclade.

Discussion

Phylogeny of *Hordeum*

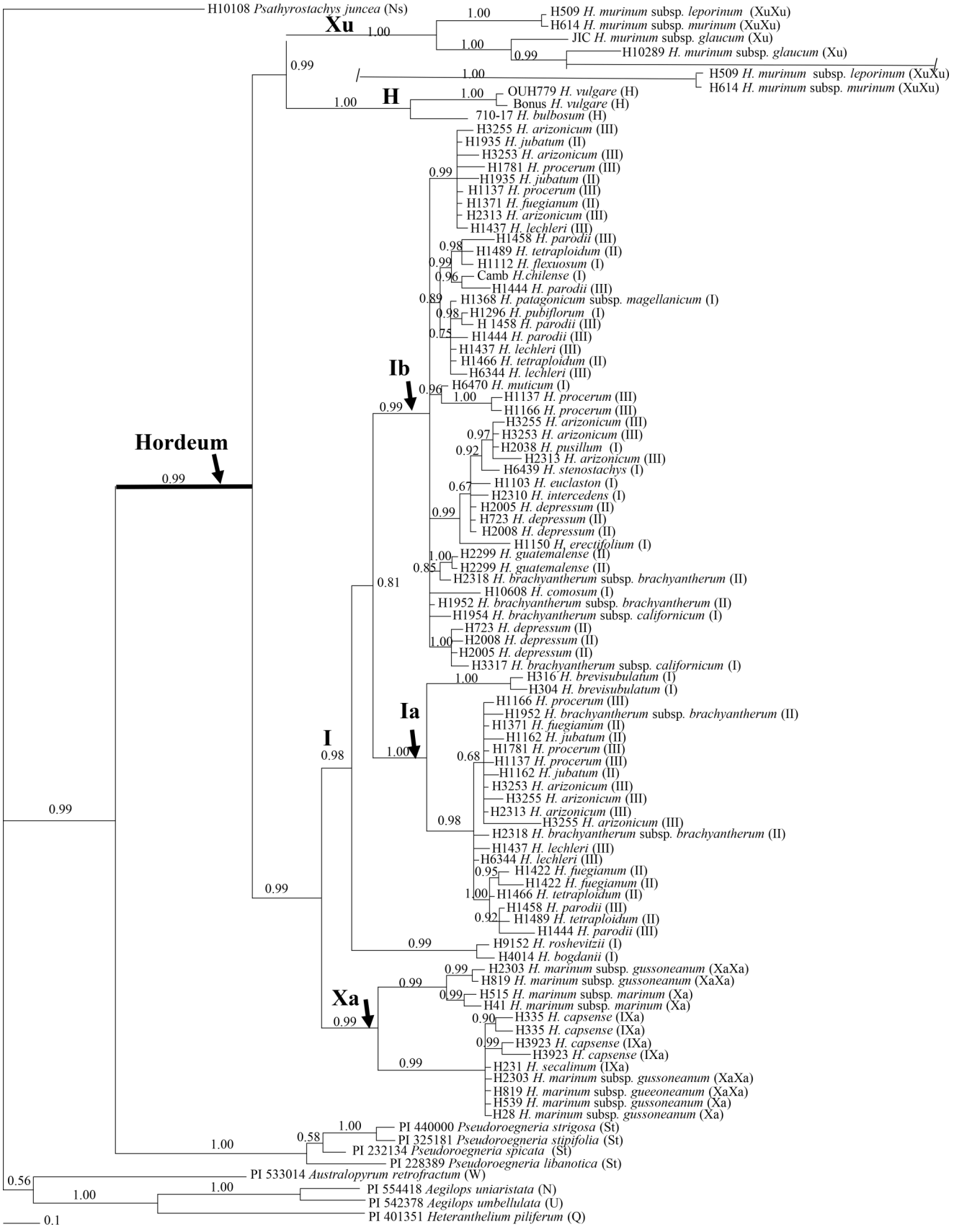
Phylogenies of diploid species of *Hordeum* derived from *RPB2* and *HTL* have previously been reported (Kakeda et al. 2009; Sun et al. 2009; Wang et al. 2011). Phylogeny of *HTL* sequences from both diploids and polyploids here revealed the origin of the genus *Hordeum* to be monophyletic. This is entirely compatible with the classification of species of *Hordeum* into four genome groups and further supports the sister relationships of **I**/**Xa** and **H**/**Xu** taxa (Komatsuda et al. 1999; Petersen and Seberg 2003; Blattner 2004, 2009; Kakeda et al. 2009; Wang et al. 2011).

The phylogenetic tree (Fig. 1) derived from *RPB2* sequences deviates from the phylogenetic tree of *Hordeum* derived from the phylogenetic tree summarized by Blattner (2009) based on previous multiple sequences data. The major difference is the non-monophyly of *Hordeum* due to the sequences from *H. murinum*, *H. procerum* (H1137), and *H. jubatum* (H1162) being placed outside the *Hordeum* clade (Fig. 1). This is not surprising as no single marker was able to consistently resolve all species relationships among the diploid species of *Hordeum* (Blattner 2009; Petersen et al. 2011). One of the possibilities is gene introgression between some polyploid species of *Hordeum* and other diploids or polyploids in Triticeae (Wang and Sun 2011; Sun and Sun 2012), resulting in *H. procerum* (H1137) and *H. jubatum* (H1162) being placed outside the *Hordeum* clade.

Origin of tetraploid IXa-genome species

A well-supported **Xa**-genome clade in both *RPB2* and *HTL* phylogenetic trees was obtained, which contains only sequences from the species with **Xa** genome. Both *RPB2* and *HTL* phylogenetic trees grouped tetraploids *H. capense* and *H. secalinum* with diploids *H. marinum* subsp. *marinum* and *H. marinum* subsp. *gussoneanum* together, confirming the presence of **Xa** genome in *H. capense* and *H. secalinum* (Taketa et al. 1999, 2009; Blattner 2004, 2006;

Fig. 2. Consensus trees derived from *HTL* sequence data using Bayesian analysis. Numbers above branches are Bayesian posterior probability (PP) values. *Psathyrostachys juncea* was used as the outgroup. The number preceding the species name is the taxa accession number.



Petersen and Seberg 2004; Brassac et al. 2012; Brassac and Blattner 2015). *Hordeum marinum* subsp. *marinum* was proposed as the progenitor of these two polyploids by Jakob and Blattner (2006) and Taketa et al. (2009), while other studies favored *H. marinum* subsp. *gussoneanum* as the progenitor of these two polyploids (Petersen and Seberg 2004; Brassac et al. 2012; Brassac and Blattner 2015). Genomic in situ hybridization indicated that *H. secalinum* has a polyphyletic origin, some populations originating from *H. marinum* subsp. *gussoneanum*, others originating from *H. marinum* subsp. *marinum*, and *H. capense* has *H. marinum* subsp. *gussoneanum* as the **Xa**-genome donor (Taketa et al. 2009). Our data could not discern which diploid as the **Xa**-genome donor to these two tetraploids, instead, indicated that either *H. marinum* subsp. *marinum* or *H. marinum* subsp. *gussoneanum* contributed to the formation of these polyploids, and favoring polyphyletic origin hypothesis of *H. secalinum* and *H. capense* (Blattner 2009). Petersen and Seberg (2004) suggested that the **I** genome in *H. capense* and *H. secalinum* was contributed by *H. brevisubulatum*. Taketa et al. (2009) found that the typical rDNA pattern of the **I** genome in *H. capense* and *H. secalinum* did not match the rDNA patterns of any Central Asian diploid species of *Hordeum*, rather they were similar to those of the "chilense type" species in the American Continents. *TOPO6* sequence suggested that an extinct diploid progenitor belonging to the Central Asian group of species of *Hordeum* contributed the **I** genome to these tetraploids (Brassac et al. 2012). Brassac and Blattner (2015) suggested that the **I** genome in *H. capense* and *H. secalinum* might be contributed by an extinct diploid species. One type of *RPB2* sequence from the two tetraploids was sister to the group containing diploid *H. pubiflorum* and *H. patagonicum* complex species, suggesting that diploid *H. pubiflorum* and *H. patagonicum* complex species might be the **I**-genome donor to the two tetraploids, which is congruent with the phylogenetic tree of *Hordeum* summarized from multiple phylogenetic and cytological studies by Blattner (2009), and strongly supports a South American origin of one of the *H. secalinum*/*H. capense* progenitors (Baum and Johnson 2003). We could not identify the other genome of *H. secalinum* and *H. capense* based on *HTL* data. Only one copy of *HTL* sequence was successfully recovered from *H. secalinum*, and two copies from *H. capense* were clustered into the same group. Previous studies also reported only one copy of *ITS* and *EF-G* in *H. secalinum* (Blattner 2004; Komatsuda et al. 2009), suggesting that two genomes in *H. secalinum* might be too similar to each other. The possibility of either the cloning procedure or some rearrangement within the genomes after intergenomic polyploidization (Taketa et al. 2009) also cannot be excluded.

American diploid solely contributes to the origin of *H. depressum*

It is consistently agreed that *H. depressum* originates from American diploid *H. californicum* (Doebley et al. 1992; Taketa et al. 2005; Jakob and Blattner 2006; Komatsuda et al. 2009; Wang and Sun 2011; Brassac et al. 2012; Brassac and Blattner 2015) together with other American diploids, either *H. euclaston* or *H. intercedens* (Blattner 2004; Wang and Sun 2011; Brassac et al. 2012), and either *H. intercedens* or *H. pusillum* (Taketa et al. 2005). The data of Komatsuda et al. (2009) and Brassac and Blattner (2015) favored *H. intercedens*, which was also supported by our *RPB2* data. In our *HTL* phylogenetic analysis, one type of *H. depressum* sequence was clustered with *H. euclaston*, *H. intercedens*, *H. pusillum*, and *H. stenostachys* with 0.99 PP (Fig. 2), pinpointing these taxa as potential parental species. Considering our *RPB2* and *HTL* phylogenies, we favor that *H. depressum* originates from the American diploids *H. californicum* and *H. intercedens*. Our results further confirm that *H. depressum* is the "purely" American tetraploid species (Brassac et al. 2012).

Asian diploids contribute to American tetraploid species of *Hordeum* with II genomes

Six American **II** genomic tetraploid species were studied. One type of *RPB2* sequence from the American tetraploids *H. brachyantherum* subsp. *brachyantherum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum*, and *H. tetraploidum*, except *H. depressum*, formed a group, which is sister to the Asian diploid *H. bogdanii* (PP = 0.94, Fig. 1). The second type of *RPB2* sequence from these tetraploids nested in the clade with the sequences from American diploids. Similarly, one type of *HTL* sequence from these five tetraploids clustered with the Asian diploid *H. brevisubulatum* (Fig. 2; BS = 073%, PP = 0.99). The second type of *HTL* sequence from these tetraploids clustered with different American diploid species of *Hordeum*. These results highlighted that Asian diploids have contributed to the origin of American tetraploids. ITS sequences (Blattner 2004), FISH pattern and RFLP (Taketa et al. 2005), and *TOPO6* (Brassac et al. 2012) suggested that Asian diploid *H. roshevitzii* was one of the parents to these American tetraploids. The *DMC1* data (Wang and Sun 2011) did not support the contribution of *H. roshevitzii* to these American polyploids. Surprisingly, our *RPB2* and *HTL* sequences also did not indicate the contribution of *H. roshevitzii* to the origin of these American tetraploids. Next-generation sequencing and in silico cloning of multiple nuclear loci suggested that *H. fuegianum* and *H. tetraploidum* originated from *H. pubiflorum* and an extinct species, and *H. brachyantherum* subsp. *brachyantherum*, *H. guatemalense*, and *H. jubatum* originated from two extinct species (Brassac and Blattner 2015), which also did not favor the contribution of *H. roshevitzii* to the origin of these American tetraploids. *Hordeum bogdanii*, *H. brevisubulatum*, and *H. roshevitzii* were suggested as potential parental species contributing to the origin of these American polyploids by different researchers (Blattner 2004; Taketa et al. 2005; Wang and Sun 2011; Brassac et al. 2012; Brassac and Blattner 2015; present study). Since three Asian diploids, *H. bogdanii*, *H. brevisubulatum*, and *H. roshevitzii*, were closely related to

each other (Blattner 2006), ancient species related to these three diploids might contribute to the formation of American polyploids, then differentiated at different rates in different regions of the genome, which make identification of extant diploids as parental species difficult. Geographically, it was suggested that *Hordeum* originated from south-west Asia and then migrated to America, resulting in the initial establishment of *bogdanii*-like diploids of *Hordeum* on the North America continent (Blattner 2006). Based on this suggestion, we favor that Asian *H. bodganii* or its close relative not only contributes to the origin of the American diploid species as suggested by Brassac et al. (2012), but also contributes to the formation of these American polyploids.

Origin of hexaploid species

Four hexaploids (*H. arizonicum*, *H. lechleri*, *H. parodii*, and *H. procerum*) were phylogenetically analyzed using *RPB2* and *HTL* sequences. One type of *RPB2* sequence in *H. procerum* and *H. parodii* clustered with sequences from tetraploids *H. brachyantherum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum*, *H. tetraploidum*, and diploid *H. bogdanii*. The *HTL* phylogeney also revealed a close relationship of *H. procerum* and *H. parodii* with these five tetraploids. Our data did not contradict the suggestion that *H. parodii* originated from the tetraploid species *H. tetraploidum* (Linde-Laursen et al. 1990; Blattner 2004; Taketa et al. 2005). The relationship of hexaploid *H. arizonicum* with the five tetraploids viz. *H. brachyantherum*, *H. fuegianum*, *H. guatemalense*, *H. jubatum*, and *H. tetraploidum* was not clearly revealed by *RPB2* data (Fig. 1). The *HTL* sequences indicated a close relationship between *H. arizonicum* and two tetraploids, *H. fuegianu* and *H. jubatum*. One type of *HTL* sequence of *H. arizonicum* clustered with diploid *H. pusillum*, indicating *H. pusillum* as a parental species, most likely as the maternal parent, as suggested by Nishikawa et al. (2002), to *H. arizonicum*. This finding is in accord with Rajhathy and Symko (1966) who suggested *H. arizonicum* originated from diploid *H. pusillum* and tetraploid *H. jubatum*, which was also backed up by *ITS* data (Blattner 2004), FISH and RFLP patterns (Taketa et al. 2005), *TOPO6* (Brassac et al. 2012), and next generation sequencing data (Brassac and Blattner 2015).

It was suggested that *H. lechleri* originated from the tetraploid species *H. jubatum* (Taketa et al. 2005; Brassac and Blattner 2015). The connection between *H. jubatum* and *H. lechleri* was also observed in cpDNA data (Jakob and Blattner 2006). Our *RPB2* and *HTL* data did not contradict this suggestion.

Non-*Hordeum* species contribution to the evolution of hexaploid species of *Hordeum*

One type of *RPB2* sequence from *H. procerum* (H1137) and *H. jubatum* (H1162) was grouped with *Au. retrofractum* (**W**) and *Ag. cristatum* (**P**) (Fig. 1), suggesting that non-*Hordeum* species in the tribe Triticeae might contribute to the evolution of these two taxa. *DMC1* sequences also revealed **St** copy of *DMC1* (*Pseudoroegneria*) in *H. arizonicum* and **Ta** (*Taeniatherum caput-medusae*) type of *DMC1* in tetraploids *H. fuegianum*, *H. jubatum*, and *H. tetraploidum* (Wang and Sun 2011). Genetic contribution from outside the genus to the species evolution within the genus is often reported in Triticeae, and one of the probabilities might be caused by polyploidy hybridization and introgression (Mason-Gamer 2008; Mahelka and Kopecky 2010). Mason-Gamer (2004) revealed five distinct gene lineages of nuclear starch synthase gene in hexaploid *Elymus repens* with the genome **StStH** (Dewey 1984). Mahelka and Kopecký (2010) found *Hordeum*-like, *Pseudoroegneria*-like, *Taeniatherum*-like, and one that did not have close relationship with any of the diploid sampled, in *E. repens*. Such complex evolution is also expected to occur in the evolutionary history of *Hordeum*. Placement of *H. murinum* subsp. *glaucum* between the two genes was different. In the *Rpb2* tree, *H. murinum* subsp. *glaucum* was sister to **St** (*Pseudoroegneria*) and **V** (*Dasypyrum*) genome species. One sequence each from accession H1137 of *H. procerum* and accession H1162 of *H. jubatum* formed a group with *Au. retrofractum* (**W**) and *Ag. cristatum* (**P**). A possible reason might be conflicting histories due to post-hybridization gene conversion with non-*Hordeum* species, where the *RPB2* paralogs were changed to copies of the the *Hordeum*-like subgenomes.

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