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β-amylase Variation in Wild Barley Accessions

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Polymorphisms of *β*-amylase among 19 species (27 taxa, 337 accessions) of wild barley, including *Hordeum vulgare* ssp. *spontaneum* (174 accessions), *H. bulbosum* (33), *H. murinum* (81), *H. marinum* (28), *H. brachyantherum* (4), *H. jubatum* (2), *H. chilense* (2) and *H. roshevitzii* (2) were studied using both isoelectric focusing (IEF) and thermostability analysis. Wide genetic variations were found. In general, the IEF patterns of *H. vulgare* ssp. *spontaneum* were markedly different from those of other wild species. Two new *β*-amylase IEF patterns (Ia and III) were observed in *H. vulgare* ssp. *spontaneum* in addition to the two patterns (I and II) found in cultivated barley (*H. vulgare* ssp. *vulgare*). In *H. bulbosum*, *H. murinum*, *H. marinum* and other wild species, 21 new IEF patterns were observed. Besides the A, B and C thermostability types reported in cultivated barley, new thermostability types (A+, A-B, B-C and C-) were frequently observed. Some accessions from *H. arizonicum*, *H. jubatum*, *H. depressum* and *H. brachyantherum* showed superior thermostability (A+). The genetic differentiation of *β*-amylase in relation to the phylogeny of genus *Hordeum* is also discussed.

Key Words: *Hordeum*, *β*-amylase, isoelectric focusing, isozyme, thermostability, wild barley.

Introduction

Barley (*Hordeum vulgare* L.) is an important crop for brewing material and the activity of the various enzymes in malt significantly affect the malting and brewing process (MacGregor 1992). β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) catalyzes the liberation of β-maltose from the nonreducing ends of starch. Therefore, β-amylase is one of the component enzymes contributing to the "diastatic power" which is an important characteristic for estimating the quality of malt for beer production. β-amylase is one of the most heat-susceptive starch degrading enzymes of barley, the thermostability of this enzyme is as important as the total activity in the brewing process. β-amylase thermostability is closely related to the fermentability parameter named "apparent attenuation limit" (Kihara *et al.* 1998, 1999). Kreis *et al.* (1988) indicated that the β-amylase genes of barley were present both on the long arm of chromosome 4H and on the short arm of chromosome 2H, and suggested that the former gene was expressed in seed tissue, while the latter gene was expressed in non-seed tissues.

We have been analyzing the β-amylase thermostability types for more than 6,700 lines of cultivated barley from different areas of the world (Kaneko *et al.* 2001a), most of the lines have been classified into type A (high thermostability), type B (medium) and type C (low). Among these lines, highly thermostable mutants and β-amylase-less mutants were found in both Chinese and Nepalese barley resources. The analyses of gene expression (Kaneko *et al.* 2000) and QTL mapping (Kaneko *et al.* 2001b), revealed that *Bmy1* locus (on the long arm of chromosome 4H) controls the thermostability of β-amylase.

The IEF patterns of these materials have been analyzed, and two main IEF patterns (pattern I and II) were identified (Kaneko *et al.* 2002). A combined analysis of β-amylase thermostability and IEF pattern indicated that the β-amylase phenotypes could be classified into four groups: A-II, B-I, B-II and C-II. Eglinton *et al.* (1998) identified three β-amylase alleles (Bmy1-Sd2-L, Bmy1-Sd1 and Bmy1-Sd2-H) in cultivated barley; the corresponding enzymes were referred to as Sd2-L, Sd1 and Sd2-H, possessing low, intermediate and high thermostability, respectively. Sd2-L, Sd1 and Sd2-H correspond to our classification of C-II, B-I and A-II.

In wild barley species *H. vulgare* ssp. *spontaneum*, which is the wild relative of cultivated barley, a larger genetic diversity has been found (Zhang *et al.* 1993, Saghai Maroof *et al.* 1995) and a wide variation of β-amylase activity has been reported (Ahokas and Naskali 1990a, 1990b). An accession with high β-amylase thermostability and a new IEF pattern has also been observed (Eglinton *et al.* 1998). However, we have limited information about β-amylase in wild barley, especially in the wild species other than *H. vulgare* ssp. *spontaneum*.

We have clarified that even the barley seeds of β-amylase-less mutants could germinate normally (Takeda *et al.* 1998). Therefore β-amylase polymorphisms in the thermostability and IEF pattern should be independent of the adaptation of barley, and adequate marker trait for the phylogenetic study of barley. The main aim of the present study was to evaluate the genetic variation of β-amylase in wild barley

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resources by thermostability and IEF analyses, and to study the phylogenetic relationship among the wild species.

Materials and Methods

Plant materials

In the present study, 337 wild barley accessions of 19 species (27 taxa) were used (Table 1). Their main origins were Afghanistan, Iran, Iraq, the former USSR, USA, Argentina and Turkey.

H. vulgare ssp. *spontaneum* and *H. bulbosum* were grown in the field and the other species were raised in a glasshouse. They were bagged for preventing possible outcross excepting self-incompatible *H. bulbosum*.

Enzyme extraction

Crude β-amylase was extracted basically according to the method reported by Kihara *et al.* (1998). Single seeds of *H. vulgare* ssp. *spontaneum* and *H. bulbosum* accessions were used for extraction, but three or more seeds of the other wild species accessions were used because of their small seed size. Well crushed barley seeds were incubated in an extraction solution (1 mg flour: 5 µl acetate buffer, 50 mM, pH 5.5, containing 10 mM dithiothreitol) at 4°C for 10–15 hours with shaking. The extract was centrifuged at 15,000 rpm at 4°C for 10 min, and the supernatant was used as the crude enzyme solution for both thermostability and IEF analysis. At least two samples were analyzed for each accession to check for variations.

Thermostability analysis

The extract of each sample was diluted 100-fold with 50 mM MOPS buffer (pH 7.1) containing 1% bovine albumin (Fraction V, Sigma Chemical Co.). Then 50 µl of diluted sample was incubated at 57.5°C for 30 min, and immediately cooled in ice water. The activities of heat-treated and untreated samples were measured using a BETAMYL Kit (Megazyme Ltd., Wicklow, Ireland), and the relative remaining activity (%) was calculated as the activity with heat treatment/activity without treatment (Kihara *et al.* 1998). Cultivated barley 'Haruna Nijo', 'Harrington' and 'Schooner' were set as control varieties of types A, B and C, respectively.

The thermostability of newly found types were exam-

Table 1. Wild barley accessions used in this study

Species	Origin[1)]	No. of accessions
H. vulgare ssp. *spontaneum* (2x)	AFG, IRQ, IRN, SSU, MRC	174
H. bulbosum (2x)	Unknown	5
H. bulbosum (4x)	IRQ, SSU, TKY	28
H. arizonicum (6x)	USA	1
H. bogdanii (2x)	PAK	1
H. brachyantherum ssp. *brachyantherum* (6x)	USA	1
H. brachyantherum ssp. *brachyantherum* (4x)	CDA	1
H. brachyantherum ssp. *californicum* (2x)	USA	2
H. capense (4x)	SAF	1
H. cordobense (2x)	ARG	1
H. chilense (2x)	CHE	2
H. depressum (4x)	USA	1
H. erectifolium (2x)	ARG	1
H. flexuosum (2x)	ARG	1
H. intercedens (2x)	USA	1
H. jubatum (4x)	GMY, SSU	2
H. marinum ssp. *gussoneanum* (4x)	IRN, IRQ, TKY	20
ssp. *gussoneanum* (2x)	CDA	1
ssp. *marinum* (2x)	ITL, IRQ	7
H. murinum ssp. *leporinum* (6x)	Unknown	1
ssp. *leporinum* (4x)	ITL, IRN, JPN	34
ssp. *murinum* (4x)	HGY, NLD	5
ssp. *glaucum* (2x)	IRN, IRQ, AFG	41
H. patagonicum ssp. *patagonicum* (2x)	ARG	1
H. roshevitzii (2x)	CHN	2
H. secalinum (4x)	SPN	1
H. stenostachys (2x)	ARG	1
Total		**337**

[1)] AFG: Afghanistan; ARG: Argentina; CDA: Canada; CHE: Chile; CHN: China; GMY: Germany; HGY: Hungary; IRN: Iran; IRQ: Iraq; ITL: Italy; JPN: Japan; MRC: Morocco; NLD: Netherlands; PAK: Pakistan; SAF: South Africa; SPN: Spain; SSU: the former USSR; TKY: Turkey; USA: United States of America.

ined by measuring the change of relative remaining activities from 47.5°C to 62.5°C at 2.5°C intervals.

Isoelectric focusing analysis

Two kinds of IEF systems were used in this analysis. In the first step of screening, the β-amylase extract was analyzed by electrophoresis using the Phastsystem and ready-made gels (PhastGEL IEF 4.0-6.5; Amersham Bioscience). The electrophoresed gels were incubated in 3% starch solution at 37°C for 15 min, followed by staining in a KI-I_2 solution for about 15 min.

In the second step, the β-amylase extract activity of selected samples measured with the BETA-AMYLASE kit was adjusted to the same level. The high resolution isoelectric focusing was performed with a Multiphor electrofocusing apparatus (LKB) (Ainsworth *et al.* 1983) on an Ampholine PAG plate (pH 4.0–6.5, Amersham Bioscience). A constant power of 1 W cm^{-1} width of gel was applied with cooling at 15°C. After prefocusing for 30 min, an applicator strip (Pharmacia LKB.SDS, 18-1002-74) was put on the surface of the gel 2 cm from the cathode, and samples (15 µl per extract) and IEF Standard (5 µl, pI range 4.45–9.6, Bio-Rad Laboratories) were applied. The electrofocusing was terminated after another 2 hours and then the gel was incubated and stained by the method described above. The IEF Standard was stained separately according to the instructions.

Results

Thermostability analysis

In the 174 accessions of *H. vulgare* ssp. *spontaneum*, 173 accessions were classified into the A, B or C type, except for one accession (OUH760, from Iraq) that showed an intermediate type between type A and type B (Table 2 and Fig. 1). After 30 minutes of heat-treatment at 57.5°C, the relative remaining activity of OUH760 was 38.6%, while the activities of standard A (Haruna Nijo), B (Harrington) and C (Schooner) were 58.7%, 26.4% and 3.6%, respectively. In this study, the highly thermostable β-amylase type Sd3, reported by Eglinton *et al.* (1998), was not observed.

The frequency distribution of thermostability types in *H. vulgare* ssp. *spontaneum* was compared with those of barley cultivars reported by Kaneko *et al.* (2001a) (Fig. 2). Type A was the majority in both *H. vulgare* ssp. *spontaneum* (53%) and cultivated barley (52%). The frequency of type B (41%) was much higher than that of type C (5%) in *H. vulgare* ssp. *spontaneum*.

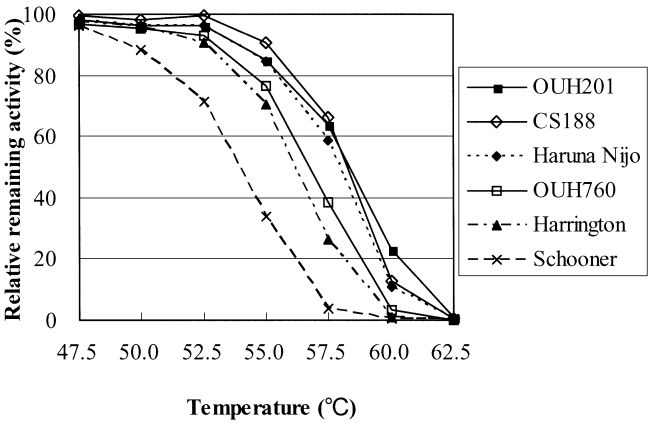

Fig. 1. Relative remaining β-amylase activity after 30 min heat treatment.

The β-amylase in the 18 *Hordeum* species showed a wider variation than that in *H. vulgare* ssp. *spontaneum*. Besides the three thermostability types (A, B and C) found in the cultivated barley, additional types such as A+, A-B, B-C and C- were frequently observed (Table 3). In these 26 taxa, 13 taxa contained type B, and only *H. bulbosum* showed the same thermostability types (A, B and C) as *H. vulgare* ssp. *spontaneum*. Type A was not observed in any wild species other than in *H. bulbosum* and *H. vulgare* ssp. *spontaneum*.

Another remarkable finding was that accessions from *H. arizonicum*, *H. brachyantherum* ssp. *brachyantherum* (4x), *H. brachyantherum* ssp. *californicum*, *H. depressum* and *H. jubatum* showed superior thermostability (A+). The relative remaining β-amylase activity of OUH201 (*H. arizonicum*) at 60°C was 23% (Fig. 1), much higher than that in 'Haruna Nijio' (A type, 11%). Between 58 and 62°C, OUH201 also showed higher relative remaining activity than CS188 which had the highest thermostability among 6,700 accessions of cultivated barley (Kaneko *et al.* 2001a). These four wild barley species could not be used directly in malting barley breeding because of the cross incompatibility. However, they could be good material for studying the thermostability mechanism.

Table 2. The diversity of β-amylase thermostability types and IEF patterns in *H. vulgare* ssp. *spontaneum*

IEF patterns	Thermostability types								Total	
	A		A-B		B		C			
I	21[1)]	(12.1)	0		45	(25.9)	0		66	(37.9)
Ia	0		0		17[1)]	(9.8)	0		17	(9.8)
II	72	(41.4)	0		9	(5.2)	9	(5.2)	90	(51.7)
III	0		1[1)]	(0.6)	0		0		1	(0.6)
Total	93	(53.4)	1	(0.6)	71	(40.8)	9	(5.2)	174	(100)

[1)] New β-amylase types which were not found in cultivated barley.
Numerals in the parentheses indicate percentage.

Table 3. The diversity of β-amylase thermostability types and IEF patterns in wild barley species

Species	Thermostability type[1)]	IEF pattern
H. vulgare ssp. *spontaneum* (2x)	**A** (93), **A-B** (1), **B** (71), **C** (9)	**I** (66), **Ia** (17), **II** (90), **III** (1)
H. bulbosum (2x)	**A** (2), **B** (2), **C** (1)	**u**[2)] (5)
H. bulbosum (4x)	**A** (20), **A-B** (4), **B** (2), **B-C** (2)	**u**[2)] (28)
H. arizonicum	**A**+(1)	**n** (1)
H. bogdanii	**B-C** (1)	**l** (1)
H. brachyantherum ssp. *brachyantherum* (6x)	**B** (1)	**d** (1)
ssp. *brachyantherum* (4x)	**A**+(1)	**n** (1)
ssp. *californicum*	**A**+(1), **B** (1)	**n** (1), **l** (1)
H. capense	**B-C** (1)	**g** (1)
H. cordobense	**B-C** (1)	**o** (1)
H. chilense	**B** (2)	**m** (2)
H. depressum	**A**+(1)	**f** (1)
H. erectifolium	**B** (1)	**o** (1)
H. flexuosum	**B** (1)	**j** (1)
H. intercedens	**B** (1)	**j** (1)
H. jubatum	**A**+(2)	**n** (2)
H. marinum ssp. *gussoneanum* (4x)	**A-B** (2), **B** (15), **B-C** (3)	**a** (9), **b** (11)
ssp. *gussoneanum* (2x)	**B** (1)	**c** (1)
ssp. *marinum*	**B** (7)	**i** (7)
H. murinum ssp. *leporinum* (6x)	**C** (1)	**p** (1)
ssp. *leporinum* (4x)	**B-C** (8), **C** (17), **C-** (9)	**q** (28), **r** (6)
ssp. *murinum*	**B-C** (2), **C** (2), **C-** (1)	**q** (3), **r** (2)
ssp. *glaucum*	**B-C** (3), **C** (16), **C-** (22)	**s** (17), **t** (24)
H. patagonicum ssp. *patagonicum*	**C** (1)	**k** (1)
H. roshevitzii	**B** (1), **C** (1)	**h** (1), **m** (1)
H. secalinum	**C** (1)	**e** (1)
H. stenostachys	**B** (1)	**o** (1)

[1)] A: high, B: medium, C: low (c.f. Fig. 2)

[2)] with small variation.

Numerals in the parentheses indicate the number of accessions.

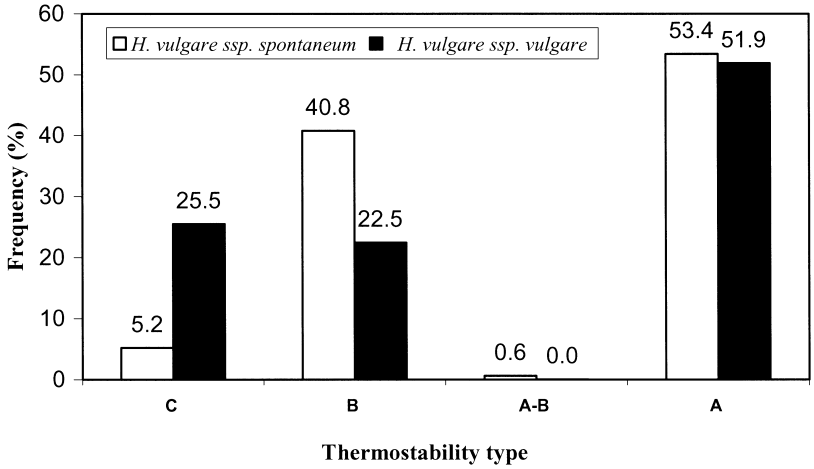

Fig. 2. Frequency distribution of β-amylase thermostability in *H. vulgare* ssp. *spontaneum* and ssp. *vulgare* (As to *H. vulgare* ssp. *vulgare*, c.f. Kaneko *et al.* 2001a).
Relative remaining activity after heat treatment at 57.5°C for 30 min.
Type C: 1~5%; B: 25~34%; A-B: 39%; A: 58~62%

IEF analysis

When the β-amylase-less varieties were tested with this method, no isozyme band was observed suggesting that only the β-amylase was detected by this method.

As a result of high resolution IEF analysis, the β-amylase zymograms in these 19 wild barley species showed a markedly wide variation (Fig. 3). The total pI range of β-amylase isozyme in wild *Hordeum* was about 4.2~6.2 and the isozyme bands at pI 4.75, 4.85 and 5.1 were the most common ones. For simplicity, the total pI range was divided

into three parts: Range I included the isozyme bands beyond pI 4.45, Range II comprised bands between pI 4.45~5.1 and Range III contained bands below pI 5.1.

Depending on the position of isozyme bands, the IEF patterns of these species could be clearly classified into four groups which contained 24 patterns (Fig. 3).

The first group included IEF patterns from **a** to **d**. Isozyme bands of these four IEF patterns were in Range I and II, almost no band was found (or very weak) in Range III. Patterns **a**, **b** and **c** were found in *H. marinum* ssp. *gussoneanum*. Patterns **a** and **b** were identified in tetraploid lines while pattern **c** was found in diploid lines. Besides *H. marinum* ssp. *gussoneanum* group, *H. brachyantherum* ssp. *brachyantherum* (6x) also showed an isozyme band at pI 4.45 (pattern **d**).

The second group included IEF patterns **e**~**o**. Isozyme bands of this group were restricted within Range II. These IEF patterns were found in *H. secalinum*, *H. depressum*, *H. capense*, *H. roshevitzii*, *H. marinum* ssp. *marinum*, *H. flexuosum*, *H. patagonicum* ssp. *patagonicum*, *H. bogdanii*, *H. chilense*, *H. arizonicum* and *H. cordobense* (Fig. 3 and Table 3). IEF pattern **e**, **f**, **g** and **h** showed two bands around pI 4.75. Two accessions of *H. roshevitzii* showed two different IEF patterns (**h** and **m**), and possessed thermostability type B and C, respectively. *H. brachyantherum* ssp. *californicum* also showed two IEF patterns (**l** and **n**) and with different thermostability types (A+ and B). It must be noted that accessions possessing IEF pattern **m** all showed superior thermostability (A+), even though they were from different taxa (*H. arizonicum* 6x, *H. jubatum* 4x, *H. brachyantherum* ssp. *brachyantherum* 4x, and *H. brachyantherum* ssp. *californicum* 2x). In addition, *H. depressum* which showed IEF pattern **f** also showed thermostability type A+.

The third group consisted of IEF patterns **p**~**u**, which showed isozyme bands in both Range II and Range III. IEF pattern **p** was observed in hexaploid *H. murinum* ssp. *leporinum*, IEF patterns **q** and **r** were found in tetraploid *H. murinum* ssp. *leporinum* and *H. murinum* ssp. *murinum*. Pattern **p** and **q** was quite similar except for the concentration difference in some isozyme bands. *H. murinum* ssp. *glaucum* showed the patterns **s** and **t**. The patterns **r** and **t** did not have the dense band at pI 4.75.

The IEF pattern of diploid and tetraploid *H. bulbosum* accessions were grouped into pattern **u**, which had the most complex band pattern in Range II. In this range, 10~12 β-amylase isozyme bands were clustered close together. There was also some small variation between the accessions and grains examined probably because of the self-incompatibility (picture not shown).

The fourth group included IEF patterns which were observed in *H. vulgare* ssp. *spontaneum*. The IEF analysis using the Phastsystem showed that the IEF patterns in

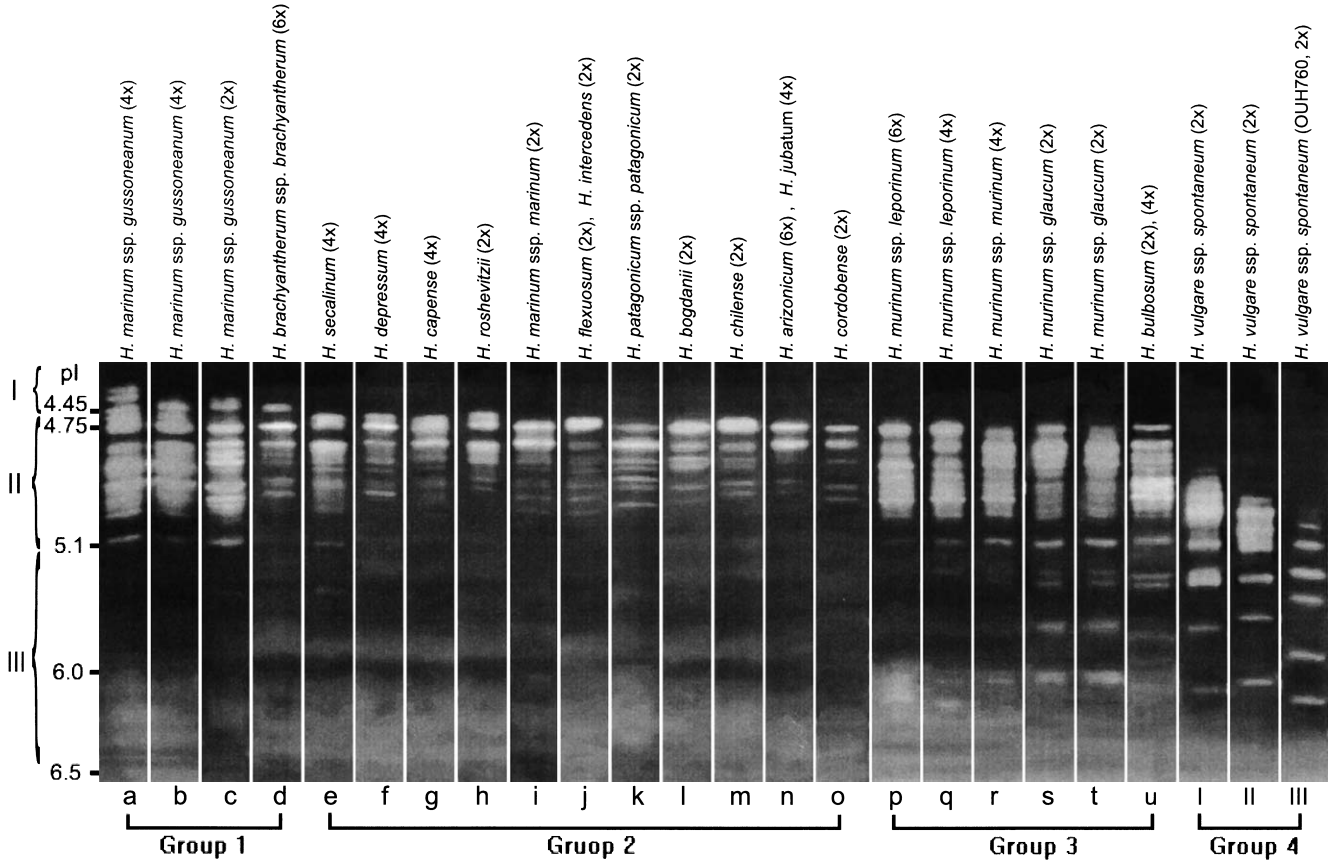

Fig. 3. IEF zymograms of β-amylase in wild barley.

H. vulgare ssp. *spontaneum* were quite similar to those in cultivated barley (Fig. 4). Besides IEF patterns **I** and **II**, a variation of pattern **I** and a unique pattern were found, they were named IEF pattern **Ia** and pattern **III**, respectively. IEF pattern **Ia** did not have the top band of pattern **I** but showed an additional band on the alkaline side. OUH760, which had an intermediate thermostability type (A-B), possessed IEF pattern **III**. The high resolution IEF analysis (Fig. 3) showed that the main bands of pattern **III** were at pI 5.1and 5.3 (the same as patterns **I** and **II**) and at pI 5.5, 5.9 and 6.2 (novel bands). The IEF patterns in *H. vulgare* did not show the isozyme bands at pI 4.75 and 4.85 which were commonly found in other wild barley species. Moreover, it was notable that *H. bulbosum* and *H. murinum* ssp. *glaucum* shared some common band positions with *H. vulgare* in Range III.

In *H. vulgare* ssp. *spontaneum*, the percentages of IEF pattern I, Ia and II were 37.9%, 9.7% and 51.7%, respectively (Table 2). While in cultivated barley patterns I and II were 15.6% and 84.2%, respectively (Kaneko *et al.* 2002). Thus, IEF pattern II was the majority in both *H. vulgare* ssp. *spontaneum* and cultivated barley.

A combined analysis of thermostability types and IEF patterns clarified that 21 (12%) and 17 (9.7%) accessions possessed β-amylase phenotype A-I and B-Ia, respectively in *H. vulgare* ssp. *spontaneum* (Table 2), these two types have not been reported in cultivated barley. Therefore, A-I, A-II, B-I, B-Ia, B-II and C-II are the six major β-amylase phenotypes in *H. vulgare* ssp. *spontaneum*. Further analysis of the DNA sequence of these typical β-amylase phenotypes should help us elucidate the structure and function of β-amylase gene.

In addition, we analyzed 43 accessions of *agriocrithon*, which had the six-rowed shattering type. These accessions showed a β-amylase variation resembling that of *H. vulgare* ssp. *spontaneum*, the six major β-amylase phenotypes (A-I, A-II, B-I, B-Ia, B-II and C-II) were all found (data not shown).

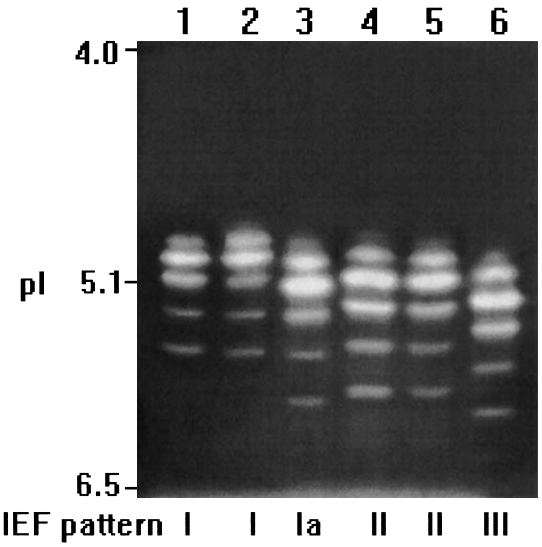

Fig. 4. Comparison of β-amylase IEF zymograms between *H. vulgare* ssp. *spontaneum* and *H. vulgare* ssp. *vulgare*.
Lane 1: 'Harrington'; Lane 2: OUH602; Lane 3: OUH689; Lane 4: 'Haruna Nijo'; Lane 5: OUH622; Lane 6: OUH760.
Lane 1,4: *H. vulgare* ssp. *vulgare*; Lane 2, 3, 5, 6: *H. vulgare* ssp. *spontaneum*

Discussion

As the wild barley is a partial outcrossing plant (*H. bulbosum* is complete outcrossing), β-amylase gene mutation will be maintained in the population in heterozygous and homozygous states. The present materials, however, were self pollinated to obtain the homozygous genotypes excepting *H. bulbosum*.

Bothmer *et al.* (1986, 1987, 1991) divided the genus *Hordeum* into four major groups with genomes **I**, **X** (**Xa**), **Y** (**Xu**) and **H**. Svitashev *et al.* (1994) used six *Hordeum vulgare* repeated nucleotide sequences (RNSs) to analyze phylogenetic relationships among 31 species (46 taxa) in the genus *Hordeum*, and the RFLP data were used to generate a phylogenetic tree for the genus *Hordeum* (Fig. 5). A comparison with our four-group classification (Fig. 3) showed that our results essentially agreed with the phylogenetic tree created by Svitashev *et al.* (1994), but with several discussible points.

The first is about the unique position of *H. bulbosum*. Dewey (1984) combined *H. bulbosum* and *H. vulgare* in one group (**I** genome) mainly based on cytogenetic data. Several reports have provided evidence supporting his hypothesis (Bothmer *et al.* 1983, 1986, 1987, Doebley *et al.* 1992, Jørgensen 1986, Kataoka *et al.* 1987, Linde-Laursen *et al.* 1990, 1992a, Pelger and Bothmer 1992). However, there are also reports that these two species were significantly different or not very closely related (Hsiao *et al.* 1986, Gupta *et al.* 1989, Molnar *et al.* 1989, Shcherban and Vershinin 1992, Xu *et al.* 1990). Molnar *et al.* (1989) suggested that the ribosomal DNA of *H. bulbosum* was more closely related to that of the *H. murinum* complex than to *H. vulgare*. Svitashev *et al.* (1994) reconfirmed the unique position of *H. bulbosum* and suggested that *H. bulbosum* and *H. vulgare* may have a monophyletic origin; the separation from the other *Hordeum* species probably occurred at a relatively early stage and later, *H. bulbosum* and *H. vulgare* diverged from their common ancestor. Komatsuda *et al.* (1999), who based their phylogenetic analysis on nucleotide sequences closely linked to the *vrs1* locus (row number of spikelets), reported that the **Xu** genome (*H. murinum* ssp. *glaucum*) and **I** genome were sister groups. In the present study, the IEF pattern of *H. bulbosum* was obviously more similar to the *H. murinum* group than to the *H. vulgare* group (Fig. 3). On the other hand, the thermostability classification of diploid *H. bulbosum* showed a high similarity to *H. vulgare*. Moreover, as mentioned above, only the IEF patterns of *H. bulbosum* and *H. murinum* ssp. *glaucum* showed similarity to *H. vulgare* in Range III. Thus, we propose that these three species probably have a common ancestor for the β-amylase gene, but

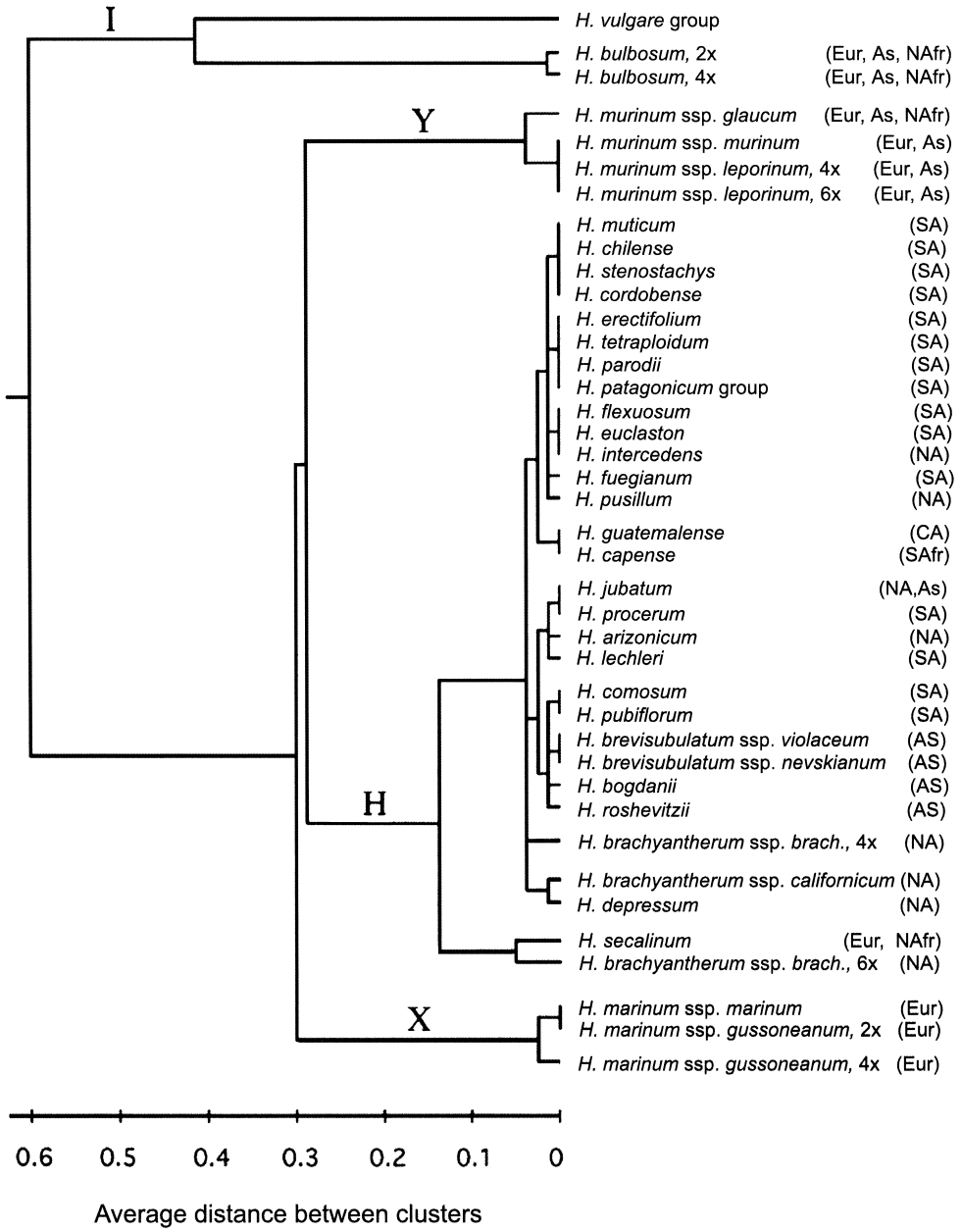

Fig. 5. *Hordeum* genus dendrogram based on the UPGMA cluster analysis (Svitashev *et al.* 1994). The geographical distribution of the species is given in parentheses. Eur: Eurpoe; As: Asia; Nafr: Northern Africa; SA: South America; NA: Northern America; CA: Central America; Afr: South Africa.

further analysis is needed to draw a conclusion.

The second point is the position of *H. marinum* ssp. *marinum* and hexaploid *H. brachyantherum* ssp. *brachyantherum*. Bothmer *et al.* (1987) proposed that *H. marinum* ssp. *marinum* and *H. marinum* ssp. *gussoneanum* are subspecies carrying the **X** (**Xa**) genome. This suggestion was supported by Linde-Laursen *et al.* (1989, 1992b), Svitashev *et al.* (1994) and Komatsuda *et al.* (1999). However, analysis of isozyme variation (Jaaska and Jaaska 1986, Jørgensen 1986, Jaaska 1992) and cpDNA diversity (Baum and Bailey 1991, Doebley *et al.* 1992) suggested a more distant relationship between *H. marinum* ssp. *marinum* and *H. marinum* ssp. *gussoneanum*. Our data is consistent with the latter hypothesis, since a very clear difference was shown between the IEF pattern of *H. marinum* ssp. *marinum* (**i**) and *H. marinum* ssp. *gussoneanum* (**a**, **b**, **c**), especially in Range I. *H. marinum* ssp. *gussoneanum* also showed more complex thermostability types (A-B, B, B-C) than *H. marinum* ssp. *marinum* (B only). Since the IEF pattern of *H. brachyantherum* ssp. *brachyantherum* (6x) also showed the isozyme band at pI 4.45, this fact may imply a close relationship between *H. marinum* ssp. *gussoneanum* and the

hexaploid *H. brachyantherum*. Doebley *et al.* (1992) reported that tetraploid *H. marinum* ssp. *gussoneanum* was most closely related to the hexaploid *H. brachyantherum*. Svitashev *et al.* (1994) reported that *H. marinum* may have been the maternal ancestor of the hexaploid *H. brachyantherum*. Nishikawa *et al.* (2002), who based molecular phylogenetic analysis on sequence data of three chloroplast DNA regions, demonstrated that the formation of hexaploid *H. brachyantherum* involved hybridization between tetraploid *H. brachyantherum* and diploid *H. marinum* ssp. *gussoneanum*. Our data support this proposition.

Svitashev *et al.* (1994) reported that *H. brachyantherum* ssp. *californicum* was much more similar to *H. depressum* than to the tetra- and hexaploid cytotypes of *H. brachyantherum*. In the present study, two accessions of *H. brachyantherum* ssp. *californicum* showed two different IEF patterns and quite different thermostability types (Table 3). One accession (H3317) showed the same superior thermostability (A+) as that of *H. depressum*, but the IEF patterns were different. The IEF pattern and thermostability type of H3317 was the same as that of *H. brachyantherum* (4x), *H. arizonicum* (6x) and *H. jubatum* (4x). Nishikawa *et al.* (2002) proposed that the formation of three tetraploids, *H. brachyantherum*, *H. jubatum* and *H. guatemalense*, probably involved hybridization between *H. brachyantherum* ssp. *californicum* and an altered **H** genome diploid. The formation of *H. arizonicum* involved the two taxa *H. jubatum* and *H. pusillum*. Based on this information and our data, it is reasonable to presume that the character of superior thermostability in *H. jubatum*, *H. arizonicum* and tetraploid *H. brachyantherum* might derive from *H. brachyantherum* ssp. *californicum*.

Over all, our analysis indicated that β-amylase IEF zymograms together with thermostability types reflected the evolution of genus *Hordeum* from one aspect. The β-amylase IEF zymogram in *H. vulgare* is distinctly different from that in other wild species. The main differentiation of β-amylase preceded the domestication of barley, since in *H. vulgare* ssp. *spontaneum* a wider genetic variation was found than that in *H. vulgare* ssp. *vulgare*. Our data also demonstrated that 14 out of 27 taxa contained thermostability type B; and, type A was not observed in any wild species other than in *H. bulbosum* and *H. vulgare* ssp. *spontaneum* (Table 3). Therefore, we suggest that type B was the most basic type probably being the prototype of β-amylase thermostability in genus *Hordeum*.

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