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A Phylogenetic Analysis Based on Nucleotide Sequence of a Marker Linked to the Brittle Rachis Locus Indicates a Diphyletic Origin of Barley

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• *Background and Aims* Barley (*Hordeum vulgare* ssp. *vulgare*) cultivation started between 9500 and 8400 years ago, and was a major part of ancient agriculture in the Near East. The brittle rachis is a critical trait in the domestication process.
• *Methods* A DNA sequence closely linked to the brittle rachis complex was amplified and resequenced in a collection of cultivated barleys, wild barleys (*H. vulgare* ssp. *spontaneum*) and weedy brittle rachis varieties (*H. vulgare* ssp. *vulgare* var. *agriocrithon*). The sequence was used to construct a phylogenetic tree.
• *Key Results* The phylogeny separated the W- (*btr1*-carrying) from the E- (*btr2*-carrying) cultivars. The wild barleys had a high sequence diversity and were distributed throughout the W- and E-clades. Some of the Tibetan var. *agriocrithon* lines were closely related to the E-type and others to the W-type cultivated barleys, but an Israeli var. *agriocrithon* line has a complex origin.
• *Conclusions* The results are consistent with a diphyletic origin of barley. The W- and E-type cultivars are assumed to have evolved from previously diverged wild barley via independent mutations at *Btr1* and *Btr2*.

Key words: *Hordeum vulgare*, cultivated barley, wild barley, weedy barley, var. *agriocrithon*, *btr1*, *btr2*, domestication, evolution.

INTRODUCTION

Barley (*Hordeum vulgare* ssp. *vulgare*) was one of the first crop species to be developed in the ‘Fertile Crescent’ (Zohary and Hopf, 1993). Archaeological remains of non-brittle barley grains indicate that selection by man of tough rachis forms of wild barley (*H. vulgare* ssp. *spontaneum*) was probably the initial stage of the domestication process (Harlan, 1992). In addition to the Fertile Crescent, Tibet, Ethiopia and Morocco have all been proposed as alternative candidate regions for the site of barley domestication (Åberg, 1938; Xu, 1982; Bekele, 1983; Molina-Cano *et al.*, 1987, 2005; Zohary, 1996). The six-rowed brittle Tibetan barley *H. agriocrithon* was identified by Åberg (1938), and was considered to be the progenitor of cultivated six-rowed barley (Åberg, 1940; Friesleben, 1943). However, other authorities have suggested that it was derived from a hybrid between wild barley and six-rowed cultivated barley (Zohary, 1963; Konishi, 2001; Tanno and Takeda, 2004), even though the presence in Tibet of a true ssp. *spontaneum* has yet to be established. Some further alternatives are that *H. agriocrithon* arose from a secondary mutant, or that it descends from a weedy hybridized segregant out of a hybrid between oriental and occidental-type cultivated barleys, which have diverged substantially from one another (Bothmer *et al.*, 1995). As a result, the latter authors have suggested *H. vulgare* ssp. *vulgare* var. *agriocrithon* (hereafter var. *agriocrithon*) as the proper taxonomic classification of this subspecies.

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The origin of barley remains to be resolved. Evidence has been presented that the mutation from brittle to non-brittle rachis must have occurred on at least two independent occasions (Takahashi, 1955). Supporting the hypothesis of a polyphyletic origin, Zohary (1996) opined that domestication was a multiple event. However, Badr *et al.* (2000) have suggested that the Israel–Jordan area section of the Fertile Crescent was the only place where wild barley was domesticated, proposing instead a monophyletic origin. Molecular studies of the key traits implicated in the domestication process should provide better objective evidence than studies of genes or markers which are genetically independent of the critical domestication genes for resolving the domestication question (Komatsuda *et al.*, 2004).

The brittle rachis is one of the most critical traits in the evolution and domestication of barley. In wild barley, this character is determined by two complementary genes, *Btr1* and *Btr2*, tightly linked to one another on chromosome 3H (Takahashi and Hayashi, 1964). In cultivated barleys, one or other of these has been lost by mutation. Most occidental cultivars are of genotype *btr1Btr2* and are referred to hereafter as W-type, while most oriental ones are *Btr1btr2* (E-type) (Takahashi, 1955). Using markers derived from a high-density AFLP-based genetic map based on an E-type × W-type cross, a phylogenetic analysis showed a clear separation between the E- and W-clades (Komatsuda *et al.*, 2004). The AFLP marker e09m25-08, which co-segregated with *btr1/btr2* (Komatsuda *et al.*, 2004; Senthil and Komatsuda, 2006), was converted to an STS (sequence-tagged site) format, and high-resolution mapping using this

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assay demonstrated a low level of recombination with *btr1* (0·21 cM; Azhaguvel *et al.*, 2006; Vidya Saraswathi *et al.*, 2006). The definition of sequence polymorphism in a closely linked marker (0·1 cM) was used to infer the multiple origin of six-rowed barley (Tanno *et al.*, 2002), and these conclusions have recently been verified following the isolation of the six-rowed spike gene *vrs1* (Komatsuda *et al.*, 2007). Since the non-brittle rachis genes have yet to be cloned, we have adopted a similar approach to track the genealogy of the brittle rachis gene complex in the evolution from wild to cultivated barley.

MATERIALS AND METHODS

Plant material

Twenty-three barley *Hordeum vulgare* ssp. *vulgare* L. cultivars, three accessions of *H. vulgare* ssp. *vulgare* var. *agriocrithon* (Åberg) Bowd., and 18 of wild barley *H. vulgare* ssp. *spontaneum* C. Koch. were obtained from several sources (Table 1). In addition, one line of *H. bulbosum* and one of *H. murinum* were included as potential paraphyletic outgroups for the phylogenetic study. The taxonomic treatment follows Bothmer *et al.* (1995). Genotype with respect to *btr1*/*btr2* was taken from the work of Takahashi *et al.* (1983), Komatsuda and Mano (2002) and Komatsuda *et al.* (2004). Exploratory DNA amplification and sequencing were carried out on DNA templates from ‘Azumamugi’ (AZ, E-type), ‘Kanto Nakate Gold’ (KNG, W-type) and OUH602 (wild barley).

DNA isolation, amplification and sequencing

Genomic DNA was extracted from young leaves following procedures described by Komatsuda *et al.* (1998). The e09m25-08STS-Ext sequence was amplified by the primers M679M06a620U037 (5′-AGAAGCTCACAGGG TTAGAAT-3′) or M679M06a990U073 (5′-TTGTGAAGG CTCTCCAGAGTC-3′) in combination with M679M 06a990L643 (5′-TACGAGGAGCTGGTCAAGGAA-3′) (Fig. 1). The 10-μL PCRs contained 20 ng genomic DNA, 300 nM each primer, 200 μM dNTP, 25 mM TAPS (*N*-Tris(hydroxymethyl)methyl-3-amino-propanesulphonic acid, pH 9·3), 50 mM KCl, 1 mM 2-mercaptoethanol, 2·5 mM $MgCl_2$ and 0·25 U ExTaq DNA polymerase (Takara, Tokyo). Reactions were denatured (94 °C/5 min), amplified for 30 cycles of 94°C/30 s, 62 °C/30 s and 72 °C/30 s, and finally incubated at 72 °C for 7 min. Amplicons were separated by 1·8 % (w/v) agarose (Iwai Kagaku, Tokyo) gel electrophoresis, eluted from the gel and purified using the Qiaquick gel purification kit (Qiagen, USA). The purified DNAs were sequenced using the Bigdye Terminator version 3·1 (ABI, Tokyo) system.

Phylogenetic analysis

Sequence alignment was performed using CLUSTAL W (Thompson *et al.*, 1994) with manual refining. Indels (insertions and deletions) shared by two or more taxa were included as ‘01’ codes for the analysis, in addition to all nucleotide substitutions. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (1987). Trees were computed with PAUP* 4·0b10 (Swofford 1998). The confidence of each clade was estimated by bootstrap analysis using 1000 pseudo-replicates.

Recombination analyses

Two methods were used to search for intragenic recombination. The first employed the program GENECONV 1·81 (http://www.math.wustl.edu/~sawyer/geneconv/index.html) developed by Sawyer (1998). The global permutation *P* values are based on BLAST like global scores (10 000 replicates). The second involved a search for recombination using DnaSP program version 4·10·7 (Rozas *et al.*, 2003).

RESULTS

The e09m25-08STS-Ext locus is highly variable

The AZ, KNG and OUH602 e09m25-08STS-Ext sequences were highly variable (Fig. 1). The AZ fragment was 554 bp in length, the KNG one 589 bp and the OUH602 one 562 bp. The AZ and KNG sequences differed from one another by 26 single nucleotide substitutions and ten indels, and KNG and OUH602 by 30 single nucleotide substitutions and eight indels; but AZ and OUH602 differed by just four single nucleotide substitutions and four indels (Fig. 1). The sequences from ‘Morex’ and KNG were identical to one another. Both the *Mse*I and *Eco*RI recognition sites, which are responsible for the AFLP fragment e09m25-08, were present in the KNG sequence (Fig. 1), while the absence of the fragment in AZ and OUH was due to the loss of both of these sites (Komatsuda *et al.*, 2004; Senthil and Komatsuda 2006). An STS marker (e50m21-01STS) which maps 0·63 cM proximal to *btr1*/*btr2* (Azhaguvel *et al.*, 2006) was also considered but, despite an amplicon size of >1 kb, only limited polymorphism existed between the wild barley OUH602 and the cultivars AZ and KNG (data not shown).

Phylogenetic analysis based on the e09m25-08STS-Ext locus

When the original forward primer (M679M06a620U037) was replaced by M679M06a990U073, a better level of amplification efficiency and stability was achieved (Fig. 1). A single fragment was amplified from all accessions of cultivated barley, var. *agriocrithon* and wild barley (data not shown). The multiple sequence alignment generated a matrix consisting of 44 taxonomic entities and 552 nucleotide sites, of which 490 were invariant, 25 variable but parsimony-uninformative, and 37 variable and parsimony-informative. Ten phylogenetically informative indels were added to the data matrix. Although an attempt was made to use either *H. bulbosum* and *H. murinum* to provide an outgroup(s) to root the phylogenetic tree, this was not possible, because neither of these templates amplified a single species amplicon. As a result, an un-rooted tree was constructed. The resulting un-rooted tree consisted of two major clades (Fig. 2), separated with a bootstrap value of 100.

TABLE 1. *Plant materials used for the phylogenetic analysis (E,* Btr1Btr1btr2btr2*; W,* btr1btr1Btr2Btr2*)*

Taxon	Name/accession number	Origin	Phenotype	Genotype	Row type	Source*
ssp. *vulgare*	Azumamugi	Japan	Non-brittle	E	6	1
	Bonus	Sweden	Non-brittle	W	2	2
	Cairo 1 (OUB369)	Egypt, Cairo	Non-brittle	E	6	3
	Caveda	Spain	Non-brittle	W	6	3
	Chevalier	UK	Non-brittle	W	2	4
	Debre Zeit 29	Ethiopia	Non-brittle	W	2	4
	Dissa	Germany	Non-brittle	W	6	5
	Esfahan 1 (OUI032)	Iran, Esfahan	Non-brittle	E	6	3
	Goheung Covered 1 (OUK001)	South Korea, Goheung	Non-brittle	E	6	3
	Golden Promise	UK	Non-brittle	W	2	4
	Hanna	Czechoslovakia	Non-brittle	W	2	4
	Haruna Nijo	Japan	Non-brittle	W	2	3
	Hayakiso 2	Japan	Non-brittle	E	6	3
	Kanto Nakate Gold	Japan	Non-brittle	W	2	1
	Kristina	Sweden	Non-brittle	W	2	2
	Misato Golden	Japan	Non-brittle	W	2	1
	Morex	USA	Non-brittle	Unknown	6	6
	Natsudaikon Mugi	Korea	Non-brittle	W	6	4
	New Golden	Japan	Non-brittle	W	2	1
	Pukou 1 (OUC018)	China, Pukou	Non-brittle	E	6	3
	Sama 1 (OUN005)	Nepal, Sama	Non-brittle	E	6	3
	Soren Oumugi 19329	Former USSR	Non-brittle	E	6	1
	Tayeh 1 (OUC331)	China, Tayeh	Non-brittle	E	6	3
var. *agriocrithon*	OUH786	Tibet, Tsela Dzong	Brittle		6	3
	OUH797	Tibet, Tsela Dzong	Brittle		6	3
	OUH802	Isreal, N.Negev	Brittle		6	3
ssp. *spontaneum*	H3140A	Cyprus	Brittle		2	7
	OUH602	Caspian Sea Reagion	Brittle		2	3
	OUH624	Afghanistan, Heart	Brittle		2	3
	OUH630	Afghanistan, Kandahar	Brittle		2	3
	OUH638	Jordan	Brittle		2	3
	OUH644	Turkmenistan, Sumbar	Brittle		2	3
	OUH707	Iraq, Karkuk	Brittle		2	3
	OUH725	Turkey, Mardin	Brittle		2	3
	OUH726	Turkey, Silvan	Brittle		2	3
	OUH728	Iran, Kermanshah	Brittle		2	3
	OUH729	Iran, Karand	Brittle		2	3
	OUH730	Turkmenistan, Karakala	Brittle		2	3
	OUH742	Iraq, Jarmo	Brittle		2	3
	OUH743	Iraq, Karkuk	Brittle		2	3
	OUH776	Morocco, Djebel	Brittle		2	3
	OUH777	Morocco, Djebel	Brittle		2	3
	OUH783	Libya, Taknis	Brittle		2	3
	PI282597	Israel, C. Israel	Brittle		2	8
H. bulbosum	H3878	Italy	Brittle		2	3
H. murinum	H74	Egypt	Brittle		2	3

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Each clade contained a mixture of wild and domesticated types. The upper clade included all (bar one) of the W-type cultivars, together with a group of wild barleys of diverse geographical origin (e.g. OUH624 from Afghanistan, OUH728 from Iran, OUH725 and OUH726 from Turkey, OUH644 from Turkmenistan, and PI282597 from Israel), while a small sub-clade linked two accessions from Iraq (OUH743) and Turkmenistan (OUH730). The Japanese cultivars fell within the W-type cluster, as expected, given that they were bred from European germplasm. The Ethiopian 'Debre Zeit 29' (a variety classified *deficiens* in some cases) also belonged to this cluster, along with one var. *agriocrithon* accession from Tibet (OUH786) (Fig. 2). The other major clade included wild barleys from Jordan (OUH638), Iran (OUH729), the Caspian Sea Region (OUH602), Iraq (OUH707 and OUH742) and Afghanistan (OUH630). A wild barley from Cyprus (H3140A) and a var. *agriocrithon* line from Israel (OUH802) were also grouped in this clade but were distantly separated from the other members. The E-type cultivars all clustered

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        M679M06a620U037>>                   M679M06a990U073>>
Morex   AGAAGCTCACAGGGTTAGAATGCTTTGAGGCTTTAATTGTGAAGGCTCTCCAGAGTCCAGACAGGGATGCAACTGTCAAG-AACAAATGGGAATGAGAGGTCAGCAAAGGGGCAGAAAAA
KNG     AGAAGCTCACAGGGTTAGAATGCTTTGAGGCTTTGATTGTGAAGGCTCTCCAGAGTCCAGACAGGGATGCAGCTGTCAAG-AACAAATGG-AATGAGAGGTCAGCAAAGGGGCAGAAAAA
AZ      AGAAGCTCACAGGGTTAGAATGCTTTGA--------TTGTGAAGGCTCTCCAGAGTCCAGACAGGGATGCAGCTGTCAAGGAACAAATGG-AATGAGAGGTCAGGAAAGGGGCAGAAGAA
OUH602  AGAAGCTCACAGGGTTAGAATGCTTTGA--------TTGTGAAGGCTCTCCAGAGTCCAGACAGGGATGCAGCTGTCAAGGAACAAATGG-AATGAGAGGTCAGGAAAGGGGCAGAAGAA
        ****************************        *********************************** ******** ********* ************* ************ **
                                            →

Morex   ACTCAAAGACAGAGGAGAAAAGGCAAGGGGCAGGAAATTAGAAGCAAAGAGTAGATTGTAGAGTAAAATGTATACTATGCACAATAGGCAGGGATTTTGCCAGTCTTTGTTCCTTATTTT
KNG     ACTCAAACAGAGGGGAGAAAAGGCAAGGGGCAGGAAATTAGAAGCAAAGAGTAGATTGTAGAGTAAAATGTATACTATGCACAATAGGCAGGGATTTTGCCAGTCTTTGTTCCTTATTTT
AZ      ACTCAAACAGAG-GGAGAAAAGGCAAAGGGCAGGAAATTAGAAGGAAAGAGTCGACTGTAGAGTAAAATGTATACTATGCACACTAGGC------------------------TTCTTTT
OUH602  ACTCAAACAGAG-GGAGAAAAGGCAAAGGGCAGGAAATTAGAATTAAAGAGTCGACTGTAGAGTAAAATGTATACTATGCACACTAGGCAGGGATTTGGCCAGTTTTTGTTCCTTCTTTT
        ************ ************* ****************  ******* ** *************************** *****                        ** ****

Morex   GGTCCCTAGTAAATTTTAC-GGAGAGGAAACAGGTTTTTT--GCAATGGGTAGCAATTATATAGTACTACTAC----------CTAAATATTTGCAGTTGGGAAGAACTAGATTAGTTCT
KNG     GGTCCCTAGTAAATTTTAC-GGAGAGGAAACAGGTTTTTT--GCAATGGGTAGCAATTATATAGTACTACTAC----------CTAAATATTTGCAGTTGGGAAGAACTAGATTAGTTCT
AZ      GGTCCCTAGTAAATTTTACCGGAGAGGAAACAGGTTTTTTTTGCAATTGGTAGCAATTATATAGTACTACTACCCCTACCTAAATATTTGCAGTTGGGGAGAACTAGATTAGTTCT
OUH602  GGTCCCTAGTAAATTTTACCGGAGAGGAAACAGGTTTTTTT-GCAATGGGTAGCAATTATATAGTACTACTACTACCCCTACCTAAATATTTGCAGTTGGGGAGAACTAGATTAGTTCT
        ******************* ********************  ***** *************************          ******************* *****************

                                               MseI
Morex   CCTTAGTTACAGAGGGATACATGT---------------TTAACGAAATGAAACTAACGCTAATACTGGAAAACGTACATCATAGATCGATCACTGCCGATACTGGAAAATGTAAAATAC
KNG     CCTTAGTTACAGAGGGATACATGT---------------TTAACGAAATGAAACTAACGCTAATACTGGAAAACGTACATCATAGATCGATCACTGCCGATACTGGAAAATGTAAAATAC
AZ      CCTTAGGTACAGAGGGGTACATGTTCAAGGGGTACATGTTCAACGAAATGAAACTAACGCTGATACTGGAAAACATATATCATAGATCGATCACTGCCGATACTGGAAAATGTAAAAGAC
OUH602  CCTTAGGTACAGAGGGGTACATGT---------------TCAACGAAATGAAACTAACGCTGATACTGGAAAACATATATCATAGATTGATCACTGCCGATACTGGAAAATGTAAAAGAC
        ****** ********* *******               * ******************** ************ ** ********* ***************************** **

Morex   TGCTCACCGAAATTGACGACGTTGGTGCAGCTCACTGGAAGTGCTTGCGCAGAACCACGGGCTGGTGTAGCTCACTGGAAGTGCTTGCGCAGAACCGCGGGCGGGGACAGCGGGTGAGCG
KNG     TGCTCACCGAAATTGACGACGTTGGTGCAGCTCACTGGAAGTGCTTGCGCAGAACCACGGGCTGGTGTAGCTCACTGGAAGTGCTTGCGCAGAACCGCGGGCGGGGACAGCGGGTGAGCG
AZ      TGTTCACCGAAATTGACCACGCTGGTGCAGCTCACTGGAAGTGCTTGCGCAGAACC----------------------------------------GCGGGCGGTGACGGCGGGTGAGCG
OUH602  TGTTCACCAAAATTGACCACGCTGGTGCAGCTCACTGGAAGTGCTTGCGCAGAACC----------------------------------------GCGGGCGGTGACGGCGGGTGAGCG
        ** ***** ******** *** **********************************                                        ******** *** ***********
                                                                                                             ←

                        EcoRI              <<M679M06a990L643
Morex   CGTGCGCGTCTCATCTGAAT-TCGGATCTCTCTGATTTCCTTGACCAGCTCCTCGTA
KNG     CGTGCGCGTCTCATCTGAAT-TCGGATCTCTCTGATTTCCTTGACCAGCTCCTCGTA
AZ      CGTGCGCGTTTCATCTAAAAGTCGGATCTCTCTGATCTCCTTGACCAGCTCCTCGTA
OUH602  CGTGCGCGTTTCATCTAAAAGTCGGATCTCTCTGATCTCCTTGACCAGCTCCTCGTA
        ********* ****** **  *************** ********************
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FIG. 1. Genomic sequence of the e09m25-08STS-Ext amplicon in Morex (BAC M679M06), AZ, KNG and OUH602. The *Mse*I and *Eco*RI restriction sites responsible for the AFLP fragment e09m25-08 are indicated in Morex and KNG. The fragment sequence is extended in both directions in the Morex BAC clone. Primers M679M06a620U037 and M679M06a990L643 were used to amplify the full sequences from the four lines. Later M679M06a990U073, instead of M679M06a620U037, was used for phylogenetic study because it generated more strong and stable PCR products than did M679M06a620U037. The sequences flanked by the arrows (552 nts) were considered for the phylogenetic analysis. Bases that are identical in all four sequences are indicated by asterisks (*).

within this clade, which also contained the Spanish 'Caveda' (a W-type cultivar) and one Tibetan var. *agriocrithon* line (OUH797). The sequence of the Moroccan (OUH776 and OUH777) and Libyan (OUH783) wild barleys was identical with that of the major E-type cultivars (Fig. 2).

Sequence analysis of var. agriocrithon *lines with cultivars*

In all, 45 SNPs (single nucleotide polymorphisms) and indels were recognized in the comparison between the sequences of the var. *agriocrithon* lines and representative E- and W-type cultivars (Fig. 3). OUH802 has a unique sequence and its origin could not be explained by recombination events between E- and W-type versions of the sequence. No recombination was detected by *Geneconv* with any of the sequence pairs based on Bonferroni-corrected Karlin–Altschul P values ($P > 0{\cdot}05$). Also no recombination was detected by *DnaSP*.

DISCUSSION

Since there are no biological barriers to hybridization between wild and cultivated barley, all wild and cultivated barleys, including var. *agriocrithon*, are deemed to belong to a single biological species (Bothmer *et al.*, 1995). Thus, as a result of gene flow, any rigorous taxonomic distinction between wild and cultivated barleys is problematical. Of the traits that differentiate the wild from the cultivated form, the foremost is brittle versus non-brittle rachis. The e09m25-08STS-ext sequence, which is tightly linked with *btr1/btr2*, exhibits a high amount of sequence diversity in wild barley, but is less polymorphic within either the E- or the W-type groups (Figs 1 and 2). This marker thus provides a legitimate handle for inferring the history of barley domestication. Phylogenetic analysis based on the marker sequence separates the E- from the W-type barley cultivars with only a single exception (Fig. 2). A phylogeny based on AFLP markers linked to *btr1/btr2* was similarly able to differentiate between the E- and W-types, while a tree constructed from AFLP loci unlinked with *btr1/btr2* showed no clear separation between them (Komatsuda *et al.*, 2004). Neither of these phylogenetic trees could resolve the wild barleys, as most clustered in the middle of the tree. In contrast, the present approach has grouped the wild lines into two clades, indicating that this marker region is probably strongly correlated with the divergence of *btr1/btr2* in the E- and W-type cultivars. If the origin of domesticated barley was a multiple event, then the E- and W-type cultivars would have derived independently from widely diverged wild barleys.

There has been a long-standing debate concerning the origin of the six-rowed, brittle var. *agriocrithon* ever since its discovery in Tibet. Interestingly, the marker sequence of one of the var. *agriocrithon* lines (OUH786)

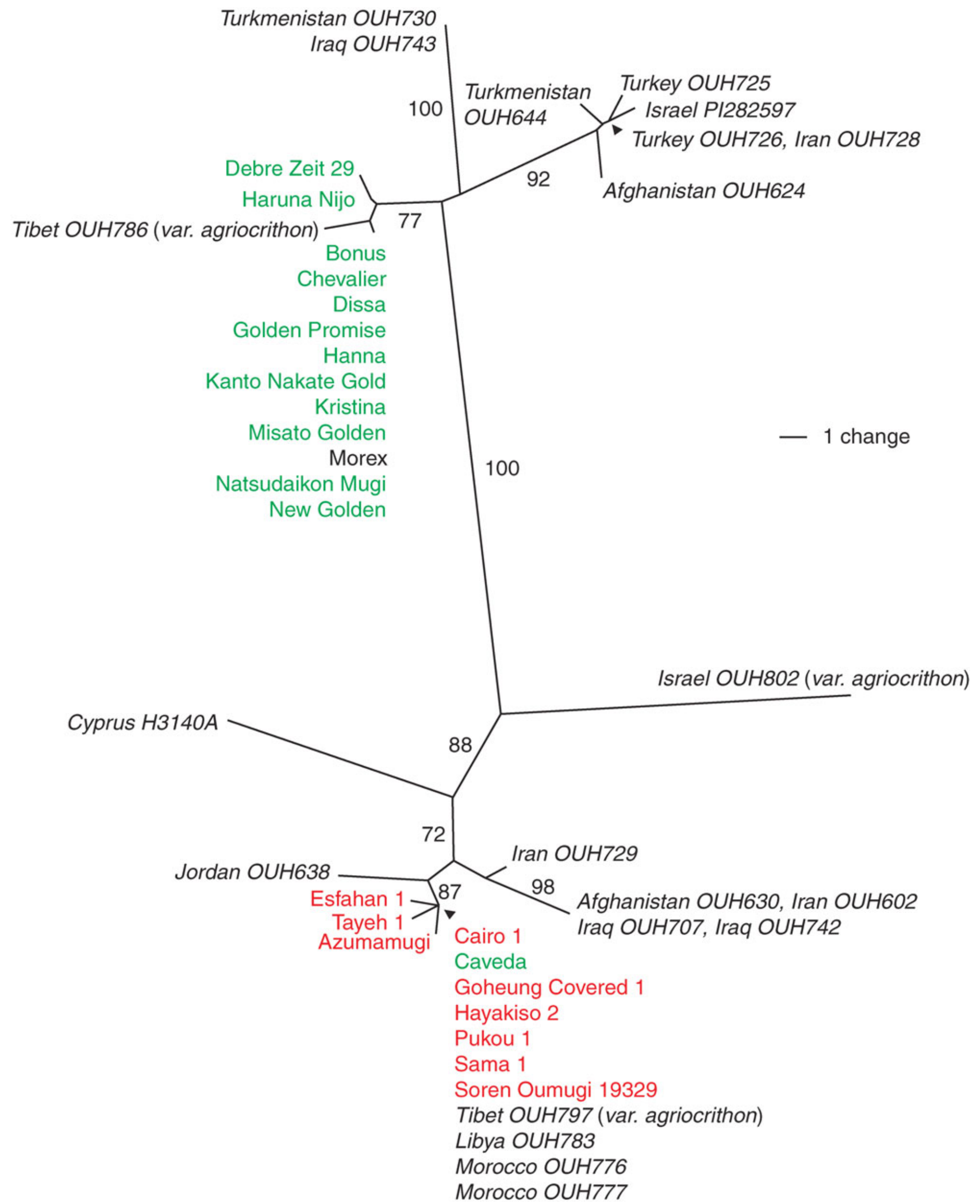

FIG. 2. Neighbor-joining tree obtained from the sequence analysis of e09m25-08STS-Ext. Wild barley lines are represented by country of origin, followed by accession numbers in italics. The three six-rowed var. *agriocrithon* lines have brittle-rachis, and are classified as *H. vulgare* ssp. *vulgare* (Bothmer and Jacobsen, 1985). Cultivated barley lines are represented in plain text. Cultivars in the upper clade shown in green are W-types (*btr1*) except for 'Morex' (unknown *btr* status). Cultivars in the lower clade shown in red are E-types (*btr2*) except for the W-type 'Caveda'. Bootstrap values with 1000 replicates >60 % are shown.

was similar to that of W-type cultivars, whereas another (OUH797) shared its sequence with some of the E-type cultivars. The cultivar × ssp. *spontaneum* origin hypothesis requires both a recombination event between e09m25-08STS-ext and *btr1*/*btr2* and the existence of wild barley in the vicinity of where cultivars are grown. Therefore, we propose a rather simpler model, based on a back mutation in the *btr1*/*btr2* region from cultivar to var. *agriocrithon* forms in Tibet. This is a likely origin for OUH797 because E-type cultivars are six-rowed, not only in the present sample but also in general (Takahashi, 1955). The origin of OUH786 may be more complicated.

Var. *agriocrithon* has frequently been also found in Israel, Cyprus and Libya (for a review, see Bothmer and Jacobsen, 1985). The Israeli var. *agriocrithon* line used here (OUH802) was well separated from both E- and W-type cultivars, as well as from all the other wild barleys and var. *agriocrithon* form (Fig. 2). Its marker sequence could not have been the outcome of a hybridization event, followed by recombination (Fig. 3). Recombination analysis by *Geneconv* and *DnaSP* did not reveal any recombination between any pairs of the marker sequences. Furthermore, it is unlikely that the brittle rachis of this line originated from a double recombination

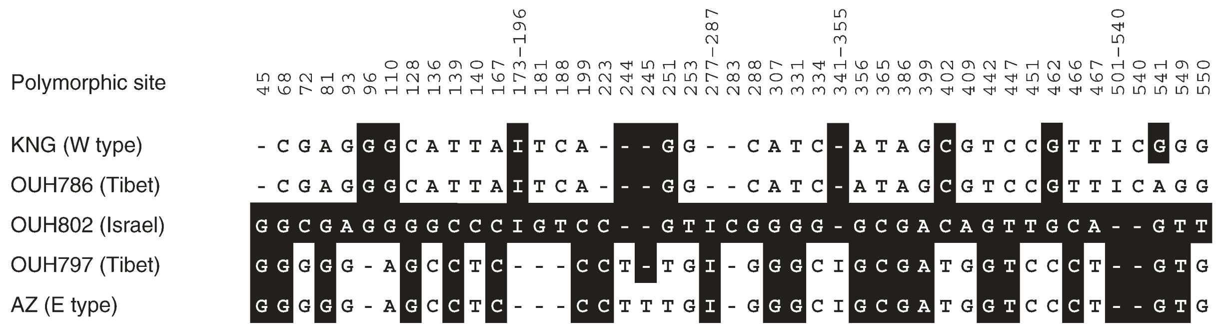

FIG. 3. Sequence comparison of three var. *agricrithon* (OUH identifiers) lines with 'Azumamugi' (E-type) and 'Kanto Nakate Gold' (W-type). The polymorphic sites in the e09m25-08STS-Ext sequence were extracted from a multiple sequence alignment. Single nucleotide substitutions and indels shared with OUH802 are indicated by shading.

event between *btr1Btr2* and *Btr1btr2* to generate a brittle *Btr1Btr2* genotype, because these two loci are tightly linked (Takahashi and Hayashi, 1964; Komatsuda *et al.*, 2004). The introgression of a six-rowed spike gene (*vrs1*) from cultivated to wild barley by outcrossing or by spontaneous mutation of *Vrs1* in wild barley may therefore have been responsible for the six-rowed spike phenotype of this form. Thus the line may represent an example of the wide ranging genetic diversity of ssp. *spontaneum*, from which it was derived. Considerable molecular diversity within var. *agriocrithon* lines has been reported (Tanno and Takeda, 2004). At the least, however, the present study supports the view that var. *agriocrithon* lines from Tibet and Israel must have different origins (Komatsuda *et al.*, 2004).

The wild barleys as a group do not cluster with either the E- or the W-types, and there is no clear association between geographic origin and placement within the phylogenetic tree. An exception to this generality is that the Libyan and Moroccan wild accessions share complete homology with some of the E-type cultivars. This was not surprising, given that a considerable number of North African cultivated barleys are of E-type (Takahashi *et al.*, 1983). Although a close relationship appears to hold between Oriental and North African barley, it is unclear as to whether either the E-type cultivars originated from North African wild barley (Molina-Cano *et al.*, 1982, 1999) or whether the two forms share the same sequence as a result of gene flow from E-type cultivars to wild barley. However, this former scenario seems improbable, given that North Africa is so geographically distant from East Asia. It is therefore hard to argue that North African wild barley could have been the immediate ancestor of the modern E-type cultivars. Morocco has not been considered as a secondary centre of barley origin (Blattner and Badani Méndez, 2001), and it has even been suggested that the Moroccan wild barley lines are weedy (Molina-Cano *et al.*, 1982). We suppose that gene flow has resulted in 'Caveda' (and most of other Western–Mediterranean cultivars; Komatsuda *et al.*, 2005) and North African wild barleys sharing alleles specific to these regions. These wild barley lines may be in a similar taxonomical situation as Tibetan var. *agriocrithon*.

Badr *et al.* (2000) excluded the possibility of a polyphyletic origin of barley, but notably this same research group has now moved to favour a diphyletic origin (Kilian *et al.*, 2006). A polyphyletic origin is also favoured by a number of other authorities (Kolodinska *et al.*, 2004; Komatsuda *et al.*, 2004; Molina-Cano *et al.*, 2005). On the basis of our comparative sequence-based study, we suggest that at least two independent brittle rachis wild populations were involved in barley domestication, and we therefore support the notion of a diphyletic origin for cultivated barley.

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