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-Canó 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.

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 X 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
assay demonstrated a low level of recombination with \textit{btr1} (0.21 cM; Azhaguvel \textit{et al}., 2006; Vidya Saraswathi \textit{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 \textit{et al}., 2002), and these conclusions have recently been verified following the isolation of the six-rowed spike gene \textit{vrs1} (Komatsuda \textit{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 \textit{Hordeum vulgare} ssp. \textit{vulgare} L. cultivars, three accessions of \textit{H. vulgare} ssp. \textit{vulgare} var. \textit{agriocrithon} (\textit{Aber}g) Bowd., and 18 of wild barley \textit{H. vulgare} ssp. \textit{spontaneum} C. Koch. were obtained from several sources (Table 1). In addition, one line of \textit{H. bulbosum} and one of \textit{H. murinum} were included as ‘01’ codes for the analysis, in addition to all

**DNA isolation, amplification and sequencing**

Genomic DNA was extracted from young leaves following procedures described by Komatsuda \textit{et al}. (1998). The \textit{e09m25-08STS-Ext} sequence was amplified by the primers M679M06a620U037 (\textit{S’-AGAAGCTCACAGGG TTAGAAT-3’}) or M679M06a990U073 (\textit{S’-TTGTGAAGG TCTCCAGAGTC-3’}) in combination with M679M06a990L643 (\textit{S’-TACCGAGGAGCTGTCAGGAA-3’}) (Fig. 1). The 10-\muL PCRs contained 20 ng genomic DNA, 300 nm each primer, 200 \muM dNTP, 25 nm TAPS (N-Tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid, pH 9.3), 50 nm KCl, 1 mm 2-mercaptoethanol, 2.5 mm MgCl2 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 % gel electrophoresis, eluted from the gel using the Qiaquick gel purification kit (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 \textit{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 \textit{P} values are based on BLAST like global scores (10,000 replicates). The second involved a search for recombination using DnaSP program version 4.0-10-7 (Rozas \textit{et al}., 2003).

**RESULTS**

The \textit{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 \textit{MseI} and \textit{EcoRI} 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 \textit{et al}., 2004; Senthil and Komatsuda 2006). An STS marker (e50m21-01STS) which maps 0.63 cm proximal to \textit{btr1/br2} (Azhaguvel \textit{et al}., 2006) was also considered but, despite an amplicon size of >1 kb, only limited polymorphism existed within 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. \textit{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 \textit{H. bulbosum} and \textit{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.

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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).

### 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                 | Israel, N.Negev      | Brittle   | 6        | 3       |
| ssp. spontaneum      | H3140A                 | Cyprus               | Brittle   | 2        | 7       |
|                      | OUH602                 | Caspian Sea Reigion  | 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, Dijbel      | Brittle   | 2        | 3       |
|                      | OUH777                 | Morocco, Dijbel      | Brittle   | 2        | 3       |
|                      | OUH783                 | Libya, Takais        | Brittle   | 2        | 3       |
|                      | PI282597               | Israel, C. Israel    | Brittle   | 2        | 8       |
| H. bulbosum          | H3878                  | Italy                | Brittle   | 2        | 3       |
| H. maritimus         | H74                    | Egypt                | Brittle   | 2        | 3       |

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within this clade, which also contained the Spanish ‘Caveda’ (a W-type cultivar) and one Tibetan var. agriocriton 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. agriocriton lines with cultivars

In all, 45 SNPs (single nucleotide polymorphisms) and indels were recognized in the comparison between the sequences of the var. agriocriton 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 > 0.05). 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. agriocriton, 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. agriocriton ever since its discovery in Tibet. Interestingly, the marker sequence of one of the var. agriocriton lines (OUH786)
was similar to that of W-type cultivars, whereas another (OUH797) shared its sequence with some of the E-type cultivars. The cultivar /C2 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 event.  

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.
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 barley 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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