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Elucidation of the origin of "*agriocrithon*" based on domestication genes questions the hypothesis that Tibet is one of the centers of barley domestication

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Summary

Wild barley forms a two-rowed spike with a brittle rachis, whereas domestic barley has two- or six-rowed spikes with a tough rachis. As does domestic barley, "*agriocrithon*" forms a six-rowed spike; however the spike is brittle the same as wild barley, which makes the origin of *agriocrithon* obscure. Haplotype analysis of the *Six-rowed spike 1* (*vrs1*) and the *Non-brittle rachis 1 (btr1*) and *2* (*btr2*) genes was conducted to infer the origin of *agriocrithon* barley. Some *agriocrithon* barley accessions (*eu-agriocrithon*) carried *Btr1* and *Btr2* haplotypes that are not found in any cultivars, implying that they are directly derived from wild barley through a mutation at the *vrs1* locus. Other *agriocrithon* barley accessions (*pseudo-agriocrithon*), carried *Btr1* or *Btr2* from cultivated barley, thus implying that they originated from hybridization between six-rowed landraces carrying *btr1Btr2* and *Btr1btr2* genotypes followed by recombination to produce *Btr1Btr2*. All materials we collected from Tibet belong to *pseudo-agriocrithon* and thus do not support the Tibetan Plateau as a center of barley domestication. Tracing the evolutionary history of these allelic variants revealed that *eu-agriocrithon* represented six-rowed barley lineages that were selected by early farmers; once in south-eastern Turkmenistan (*vrs1.a1*) and again in the eastern part of Uzbekistan (*vrs1.a4*).

Introduction

The spike of wild barley (*Hordeum vulgare* ssp. *spontaneum*) disarticulates at maturity because it has a brittle rachis. In contrast the rachis of cultivated barley (*H. vulgare* ssp. *vulgare*) remains intact. The loss of the brittle rachis, which represents one of the so-called key "domestication traits" in barley, results from a deletion in either of *Btr1* or *Btr2*, the two tightly linked genes located on chromosome 3H (Pourkheirandish *et al.*, 2015). A second major domestication trait in barley affects the spike morphology. In

Hordeum species, the spikelets that develop along the spike nodes, are arranged in triplets. In ssp. *spontaneum*, only the central spikelet is fertile, a condition retained in cultivated two-rowed ssp. *vulgare*. The six-rowed spike, in which all three spikelets at a given rachis node are fertile, appears to have arisen at least four times independently (Komatsuda *et al.*, 2007, Saisho *et al.*, 2009) through loss-of-function mutations in the gene *Vrs1* (termed *vrs1.a1*, *a2*, *a3* and *a4*). The *vrs1.a2* and *vrs1.a3* alleles originated from two-rowed cultivars carrying the functional alleles *Vrs1.b2* and *Vrs1.b3*, respectively (Komatsuda *et al.*, 2007). However, the origins of *vrs1.a1* (the most common allele in six-rowed barley) and *vrs1.a4* are yet to be resolved.

A brittle rachis, six-rowed spike barley, named *agriocrithon* (*H. vulgare* ssp. *vulgare* f. *agriocrithon* [Åberg] Bowd.), was first described by Åberg based on material from Tibet (Åberg, 1938). The origin of this type has been hotly disputed. One view is that it was derived from a wild progenitor through a mutation in the *Vrs1* gene and that it is the progenitor of six-rowed cultivars (Åberg, 1940, Schiemann, 1951). Others have suggested that it arose following hybridization between wild ssp. *spontaneum* and a cultivated six-rowed ssp. *vulgare* (Zohary, 1963, Konishi, 2001, Tanno and Takeda, 2004), and a third hypothesis is that it segregated from an inter cultivar hybrid (Bothmer *et al.*, 1995). Now that the genes of two vs. six-rowed spike (Komatsuda *et al.*, 2007) and brittle vs. tough rachis (Pourkheirandish *et al.*, 2015) have been clarified it is possible to study the evolution of *agriocrithon* barley in the framework of *Vrs1*, *Btr1* and *Btr2*. We analyzed the allelic state of these genes in a set of 454 barley lineages with the aim of (i) determining the origin of *agriocrithon* barley, (ii) understanding the origin of six-rowed barley, and (iii) infer whether Tibet is one of the centers of barley domestication (Dai *et al.*, 2012, Dai *et al.*, 2014).

Results

Genealogical lineages within the *vrs1* locus. All the 30 *agriocrithon* accessions had a brittle rachis and a six-rowed spike to fulfill the definition of *agriocrithon*. In a first step we sequenced the *vrs1* locus in 30 *agriocrithon* accessions to assess which allele(s) is responsible for their six-rowed spike type. Analyzing these data together with the full set of alleles occurring in the panel of 454 barley accessions (Saisho *et al.*, 2009) resulted in 72 distinct *vrs1* haplotypes (Table S1 and S2). In *agriocrithon* barleys three haplotypes occur, two (Hap5 and 7) belonging to the *vrs1.a1* group of alleles found in 13 accessions, and one *vrs1.a4* type (Hap8) was found in 17 accessions (Table 1). Hap5, the basic haplotype within the *vrs1.a1* allele group, is the progenitor of eight further sub-haplotypes (Hap4, 7, 9, 11, 13, 14, 16 and 17). It was derived from Hap54 (*Vrs1.b1*; Figure 1 and 2) through a single base deletion in the third *Vrs1* exon (Komatsuda *et al.*, 2007), producing a protein truncated at the C-terminal region. Hap54 occurs in the wild barley accession ICWB181495 from Turkmenistan but was not found among tested two-rowed ssp. *vulgare* accessions. Thus, it might be lost within ssp. *vulgare* or that Hap5 arose from Hap54 in ssp. *spontaneum*.

The second six-rowed type found in *agriocrithon* barley is *vrs1.a4* - represented by Hap8 (Figure 1). Because the *vrs1.a4* allele did not show any lesions within the *Vrs1* ORF, an allelism test was performed. When crossed with *vrs1* loss-of-function type (*vrs1.a1*), F_1 hybrids showed the six-rowed phenotype which confirms that *vrs1.a4* is truly allelic to the *vrs1.a1* (Figure 2). The markedly reduced abundance of *Vrs1* transcript in *vrs1.a4* carriers has been proposed as the determinant of the six-rowed phenotype (Sakuma *et al.*, 2013). Although the causative mutation/s for *vrs1.a4* remains to be identified, the key polymorphism may reside in the regulatory elements of the gene. Hap8 (*vrs1.a4* carriers) was identified in 17 *agriocrithon* (Table 1) and 21 six-rowed ssp. *vulgare* accessions (Table S1), as well as two ssp. *spontaneum* accessions (*Vrs1.b4* carriers) collected in Turkmenistan and Uzbekistan. This is a strong indication that *vrs1.a4* may have arisen in Central Asia (Table S2). Hap8 was not found among two-rowed ssp. *vulgare* accessions implying that Hap8 either was lost within two-rowed ssp. *vulgare* or that Hap8 of *agriocrithon* arose from ssp. *spontaneum*.

Only one haplotype (Hap2, Figure 3) was found in *vrs1.a2*, defined by a single base insertion in exon 2, which induces a frameshift mutation (A40>F.S.) (Komatsuda *et al.*, 2007). This haplotype originated from Hap6 (*Vrs1.b2*), which is carried by the two-rowed cultivar 'Palmella Blue' and ssp. *spontaneum* accession ICWB181462 (Table S1 and S2). 'Palmella Blue' is a North African landrace andICWB181462 was collected in Jordan. Hap2 was found in eight six-rowed ssp. *vulgare* lines from southern Europe. We hypothesized that two-rowed Hap6 was introgressed from ssp. *spontaneum* into a two-rowed ssp. *vulgare* (like Palmella Blue), after which it mutated into six-rowed Hap2. This notion is supported by the wide dispersal of *vrs1.a2* among both North African (Saisho *et al.*, 2009) and southern European cultivars (Table S1).

The *vrs1.a3* allele is represented by Hap3 and 3.2 (Figure 3). The phylogeny implies that Hap3 was derived from Hap1 (*Vrs1.b3*) through a single nucleotide substitution in exon 2, which changed a phenylalanine to leucine at position 85 (F85>L) in the conserved homeodomain (Figure 3). Hap1 was found in ssp. *spontaneum* accession ICWB181442, as well as 45 two-rowed ssp. *vulgare* lines. Hap3 occurred in 18 six-rowed ssp. *vulgare* lines distributed in Europe, East Asia and North America, whereas ICWB181442 was collected in Jordan. Thus, it seems that Hap1 was introduced into the cultivated genepool from a ssp. *spontaneum* parent, and later mutated to Hap3 within a cultivated two-rowed type. There is no pedigree data available for Hap3 lines collected from Korea, Japan or China suggesting that these lines are landraces. Since many of the Hap3-carrying cultivated barleys were collected in East Asia, it is likely that Hap3 originated there. Otherwise a cultivar carrying Hap3 was initially introduced into East Asia where it spread across the region.

Sequence variation in the *btr1* and *btr2* loci. As *agriocrithon* types are characterized by the brittle rachis, we sequenced both *Btr* loci in our set of 30 *agriocrithon* accessions to infer the allelic composition of this trait, and analyzed them together with the sequences published previously (Pourkheirandish *et al.*, 2015) . The 2.4 Kbp *btr1* sequences consist of 86 haplotypes; 81 haplotypes of ssp. *vulgare* and ssp. *spontaneum* were described previously (Pourkheirandish *et al.*, 2015). There were ten functional *Btr1* haplotypes among the *agriocrithon* accessions (Table 1). Of eight haplotypes absent from the ssp. *vulgare* genepool, three were shared with ssp. *spontaneum*, and the other five, which were likely sub-haplotypes of these three, were unique to *agriocrithon*

(Figure 4). Of the remaining haplotypes, one (B1Hap023) was present in cultivated barleys with *Btr1btr2* genotype (named as *btr2*-type), and the other (B1Hap213) was probably derived from B1Hap023 by a 62 nt insertion in the 3’ non-coding region.

The 4.9 Kbp *btr2* sequences consist of 135 haplotypes. The 127 haplotypes present in ssp. *vulgare* and ssp. *spontaneum* were described by Pourkheirandish *et al.*, (2015). Eleven functional *Btr2* haplotypes occurred in *agriocrithon* accessions (Table 1), two of which were shared with ssp. *spontaneum*, and a further six were derivatives of the latter two, and unique to *agriocrithon* (Figure 5). The remaining three comprised: B2Hap224, which was present in some *btr1*-type cultivars (Pourkheirandish *et al.*, 2015); B2Hap267, which was specific to *agriocrithon* and likely derived from B2Hap224 through a T to C substitution in the 5’ non-coding region; and B2Hap268, which differed from B2Hap267 by G to A substitution, also in the 5’ non-coding region.

The genotypic composition of *vrs1* and *Btr1/Btr2* in *agriocrithon*

***vrs1.a1* carriers.** Six *agriocrithon* accessions (HOR 4904, 3886, 3900, 3552, 8509: Hap5 and 7269: Hap7) carrying *vrs1.a1* have variable *Btr1* and *Btr2* haplotypes, implying that their *Btr* genes are largely unrelated to one another and unrelated to those of cultivars, sharing only the *vrs1.a1* allele (Table 1). Here, these *agriocrithon* barley are defined as “*eu-agriocrithon*”. The diversity with respect to collection sites for the accessions (Azerbaijan, Turkmenistan, Israel and unknown) supports the notion that they are unlikely to be related by pedigree. Hap54 (*Vrs1.b1*), the progenitor of Hap5, is present in ssp. *spontaneum* accession ICWB181495 from Turkmenistan, which suggests that this region was the site where Hap5 first became established to form an *agriocrithon* barley. Genotypes from Azerbaijan, Israel and unknown sites could be the result of out-crossing of *agriocrithon* × wild barley.

Seven *agriocrithon* accessions (HOR 2451, 2452, 2508, 2908, 2453, 2454 and 2464: Hap5) carried B1Hap023 (the most common *Btr1* haplotype among *btr2*-type cultivars). Four of them harbored B2Hap267 and the other three B2Hap268. Both haplotypes were likely derived from B2Hap224 (the most common *Btr2* haplotype among *btr1*-type cultivars) (Figure 5). All of these seven accessions were collected in Tibet suggesting that they descended from a common progenitor. The combination of *Btr1/2* haplotypes within this group is unique (Figures 4 and 5) and does not exist in wild barley, which implies that they originated through hybridization between six-rowed *btr1*-type (*btr1Btr2*) and six-rowed *btr2*-type (*Btr1btr2*) cultivars followed by recombination that produced a six-rowed type with brittle rachis (Figure 6). Here, these *agriocrithon* barley are defined as “*pseudo-agriocrithon”* (Table 1).

***vrs1.a4* carriers.** Of 17 *agriocrithon* accessions harboring *vrs1.a4* (Hap8), six (HOR 2268, 2456, 2460, 2461, 2465 and 2466) were collected in Tibet and carried B1Hap210 and B2Hap224 implying a close relationship among them. B1Hap210 is similar to B1Hap097 (Figure 4) harbored by Jordan (ICWB180007, ICWB180014, ICWB181212, and ICWB181215) and Syrian (ICWB181562 and ICWB181746) ssp. *spontaneum* accessions, whereas B2Hap224 found in *btr1*-type cultivars but not in any ssp. *spontaneum* accessions. The haplotype combination of B1Hap210 and B2Hap224 or their close relatives is not found in any of 505 wild barley accessions analyzed previously (Pourkheirandish *et al.*, 2015). Lack of the haplotype combination suggests that this six *agriocrithon* lines could be generated due to hybridization between wild and domesticated types (*Btr1Btr2* × *btr1Btr2*) followed by recombination between *Btr1/2* loci. These *agriocrithon* barley are *pseudo-agriocrithon* (Table 1).

HOR 3904 and 3906 both carry B1Hap084 and B2Hap098, a combination that also occurs in Uzbek ssp. *spontaneum* accession ICWB181752. HOR 3905 and 3907 both harbor B1Hap084 / B2Hap269; the latter haplotype was likely derived from B2Hap098 (Table 1). The Uzbek wild accession harbors Hap8 for the *vrs1.a4* allele, the same as the four *agriocrithon* accessions (Figure 3). Even though the collection sites for HOR 3904 to 3907 are unknown the evidence is strong that the Uzbek accession represents a progenitor of this small group of *agriocrithon* entries. Two accessions (HOR 3902 and 3903) carried B1Hap065 and B2Hap270. B1Hap065 was also present in four ssp. *spontaneum* accessions from Israel [FT161-163 and 647, see Supplementary Table 7 in (Pourkheirandish *et al.*, 2015)] whereas B2Hap270 is closely related to the haplotype present in two other Israeli ssp. *spontaneum* accessions [FT118 and 121, see Supplementary Table 7 in (Pourkheirandish *et al.*, 2015)]. The collection sites of HOR 3902 and 3903 are not recorded, but the haplotype data are suggestive of origins in the western side of the Fertile Crescent. These six *agriocrithon* barley are *eu-agriocrithon*

(Table 1).

Three accessions (HOR 2463, 3901 and 9517) carried B1Hap023 and B2Hap267. HOR 2507 carried B1Hap023 and B2Hap268, and HOR 1645 carried B1Hap213 and B2Hap267 (Table 1). Haplotypes at *Btr1* and *Btr2* show that both HOR 2507 and HOR 1645 were derived from three accessions (HOR 2463, 3901 and 9517). B1Hap023 is found also in a number of *btr2*-type cultivars, whereas B2Hap267 and B2Hap268 were both derived from B2Hap224 dominant in *btr1*-type cultivars, implying their origin was hybridization between cultivated forms followed by recombination between *Btr1/2* loci. HOR 1645, 2463, 2507 and 9517 are recorded as having been collected in Tibet, whereas HOR 3901 is of unknown origin. The combinations of *Btr1* and *Btr2* haplotypes represented among *agriocrithon* accessions carrying *vrs1.a4* are similar to those displayed by the Tibetan *vrs1.a1 agriocrithon* accessions, suggesting that they too arose from crosses between different ssp. *vulgare* types (*pseudo-agriocrithon*, Figure 6).

Discussion

The likely donor of *vrs1.a1* and *vrs1.a4* of ssp. *vulgare* was *agriocrithon*. A spontaneous mutation at the *vrs1* locus converted the two-rowed spike of ssp. *spontaneum* barley into a six-rowed spike which was defined as *eu-agriocrithon*. The *vrs1* gene tree shows that the *a1* and *a4* alleles, which persist in contemporary *agriocrithon*, are derived from two independent mutations. The former was likely derived from *Vrs1.b1* by a 1 bp deletion in the coding sequence (Komatsuda *et al.*, 2007). *Vrs1.b1* has been retained in ssp. *spontaneum* but not in two-rowed ssp. *vulgare.* The suggestion is that the mutation from *Vrs1.b1* to *vrs1.a1* in ssp. *spontaneum* initiated the new lineage denoted here as *eu-agriocrithon* (Figure 1). A reverse mutation (i.e., the restoration of the deleted nucleotide) would be a highly improbable event, so the likelihood of a *vrs1.a1* carrier being the immediate ancestor of *Vrs1.b1* is remote. It is not possible to exclude the possibility that mutation of *Vrs1.b1* to *vrs1.a1* occurred in a two-rowed ssp. *vulgare* line. This could have been too rare to be captured in the global germplasm set, or is possibly extinct. However there is no supportive evidence for this scenario, thus the most likely situation is that *eu-agriocrithon* was the progenitor of *vrs1.a1* six-rowed barley. The combined haplotypes at *vrs1*, *btr1* and *btr2* suggest a plausible hypothesis that *vrs1.a1 eu-agriocrithon* arose from a ssp. *spontaneum* population similar to the one from which ICWB181495 was collected (in southwest of Turkmenistan along the Iranian border in central Asia).

Both *vrs1.a4* and *Vrs1.b4* alleles share the same coding sequence and the two alleles must differ in their regulatory regions. *Vrs1.b4* is represented in ssp. *spontaneum* but not in any two-rowed ssp. *vulgare* accession hence suggesting that *vrs1.a4* arose in a wild population. Based on these data, a yet unknown mutation in *Vrs1.b4* might have generated the *vrs1.a4* allele to create another *eu-agriocrithon*. According to the combined haplotypes at *vrs1*, *btr1* and *btr2*, it is most likely that the *vrs1.a4 eu-agriocrithon* was derived from a ssp. *spontaneum* population similar to the one from which ICWB181752 was collected in the eastern part of Uzbekistan. Although an alternative scenario is that a *vrs1.a4* six-rowed ssp. *vulgare* type gained the brittle rachis trait. The probability of the necessary reverse mutation, entailing the insertion of either one nucleotide into the *btr1* allele or 11 nucleotide into *btr2,* is very low. The *Btr1* and

Btr2 haplotypes of the *vrs1.a1* and *vrs1.a4 eu-agriocrithon* accessions are rather distantly related to those found in the ssp. *vulgare* genepool, implying that *eu-agriocrithon* was unlikely to have arisen from ssp. *vulgare*. All of the *eu-agriocrithon* accessions appear to be sister forms of ssp. *spontaneum,* in which the *vrs1* gene has undergone a loss-of-function mutation. Hybridization between primary *eu-agriocrithon* and the other wild barley genotypes would generate secondary *eu-agriocrithon* forms represented by the *agriocrithon* accessions with largely unrelated *Btr1/2* haplotypes while, sharing only their *vrs1* allele.

The best current hypothesis holds that both *vrs1.a1* and *vrs1.a4* were introgressed from *eu-agriocrithon* into other wild barley genotypes and then outcrossed to cultivated barley (Figure 1). Out-crossing occurs in ssp. *spontaneum* at a frequency of around 2% (Abdel-Ghani *et al.*, 2004). Thereafter, segregation at *vrs1* and *btr1/2* could generate the combination of non-brittle rachis and a six-rowed spike, because the *vrs1* and *btr1/2* map to different chromosomes (Pourkheirandish and Komatsuda, 2007). The alternate scenario requires a mutation at one of the *Btr1* or *Btr2* genes in *eu-agriocrithon* to generate a non-brittle rachis and six-rowed spike (cultivated barley) – an event that is unlikely given the genetic distance of the *Btr1* and *Btr2* haplotypes in *eu-agriocrithon* from those in ssp. *vulgare*. Moreover, both of the mutations from *Btr1* to *btr1* and *Btr2* to *btr2* are believed to have occurred in the Levant (Pourkheirandish *et al.*, 2015), which is geographically far away from Turkmenistan / Uzbekistan.

The outcome of recombination between *btr1* and *btr2*. That recombination explains the origin of *agriocrithon* barley has been frequently suggested, but as yet no experimental demonstration has been produced to support it. A sub-group of the *agriocrithon* accessions harbor a functional *Btr1* haplotype B1Hap023 that present in *btr2*-type cultivars plus a functional *Btr2* haplotype B2Hap267 or B2Hap268 derived from B2Hap224 that present in *btr1*-type cultivars (Table 1), suggesting a recombination between genes *Btr1* and *Btr2* following cultivar hybridization (*btr1Btr2* × *Btr1btr2*) (Figure 6A). Brittle-rachis segregants have been successfully generated from inter cultivar crosses (Pourkheirandish *et al.*, 2015), showing the possibility of recombination between *btr1* and *btr2* and supporting the possible origin of *agriocrithon* through recombination. An appropriate nomenclature for such types is *pseudo-*

agriocrithon (Figure 6). Origin for this *agriocrithon* group is not through loss-of-function mutation at *vrs1* in a ssp. *spontaneum* background because there is no example of a ssp. *spontaneum* accession carrying the same *Btr1/2* haplotype as that represented among *pseudo-agriocrithon* accessions. Another sub-group of the *agriocrithon* accessions such as HOR 2456 from China, Tibet/Lhasa (Table 1) harbor a functional *Btr1* haplotype B1Hap210 that was similar to wild collected from Jordan plus a functional *Btr2* haplotype B2Hap224 that present in *btr1*-type cultivars, suggesting a recombination between genes *Btr1* and *Btr2* following wild-cultivar hybridization (*Btr1Btr2* × *btr1Btr2*) (Figure 6B). It's also very unlikely that this group is a result of loss-of-function mutation at *vrs1* in a ssp. *spontaneum* background because the haplotype combination or their close relatives does not exist in any of 505 ssp. *spontaneum* studied previously (Pourkheirandish *et al.*, 2015). The present germplasm panel included 18 *pseudo-agriocrithon* accessions, all collected from Tibet. *Agriocrithon* barley of Tibet was suggested as a progenitor for domestication, resulting in an independent type of cultivated barley (Dai *et al.*, 2012, Dai *et al.*, 2014), however this hypothesis was based on phylogenetic analysis using whole genome and transcriptome data that can be affected by recombination. Based on our current results all of the *agriocrithon* materials from Tibet are *pseudo-agriocrithon* that resulted from hybridization involving domesticated barleys. Even though we had no access to the specific *agriocrithon* accessions used by Dai et al. (2012, 2014), the present evidence suggests that Tibetan *agriocrithon* barley resulted from *btr1* and *btr2* genetic recombination that involved cultivated barley.

The origin of six-rowed barley is resolved. The proposal here is that *vrs1.a1* and *vrs1.a4* emerged in *agriocrithon* before they were introgressed into ssp. *vulgare* (Figure 1). The current germplasm panel comprised 196 six-rowed barley accessions, of which 149 were carriers of *vrs1.a1*, eight of *vrs1.a2*, 18 of *vrs1.a3* and 21 of *vrs1.a4*. The origin of two alleles (*vrs1.a2* and *a3*) was shown previously (Komatsuda *et al.*, 2007) to involve single mutations at the *vrs1* locus that changed the domesticated two-rowed to six-rowed phenotype (Figure 1). The predominant *vrs1.a1* allele included the largest number of derived haplotypes that agree with the suggestion that it represents the most ancient *vrs1* allele (Komatsuda *et al.*, 2007). Both *vrs1.a1* and *vrs1.a4* arose in Central Asia, some 2,500km distant from the site of emergence of non-brittle rachis. The occurrence of six-rowed types among wild barley implies that the six-rowed allele pre-dates domestication, when early farmers still relied on ssp. *spontaneum*. This time line agrees, in part at least, with the notion that six-rowed cultivated barley was derived from *agriocrithon* (Åberg, 1938). The non-brittle rachis trait represents a strong genetic bottleneck, and its geographical origin lies far from that of *eu-agriocrithon*, clearly implying that *eu-agriocrithon* could not have been the immediate ancestor of non-brittle barleys. They were donors of *vrs1.a1* and *vrs1.a4* to cultivated barley.

Experimental procedures

Plant materials. A set of 30 *agriocrithon* (*H. vulgare* ssp. *vulgare* f. *agriocrithon* [Åberg] Bowd.) accessions was obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany); the sites of collection included Tibet (17 accessions), Israel (2), Azerbaijan (1), Turkmenistan (1) and unknown (9) (Table 1). All these accessions had a brittle rachis and a six-rowed spike. For cultivated barley 274 accessions (Table S1) were obtained from Okayama University (Okayama, Japan), and 150 accessions of ssp. *spontaneum* of diverse geographical origins (Table S2) was provided from ICARDA (Aleppo, Syria). The material was all autumn sown in the field at NIAS (Tsukuba, Japan). Azumamugi (AZ) is a Japanese six-rowed cultivar and SV169 and SV176 are six-rowed landraces collected in Nepal were used for allelism test of six-rowed trait. GenBank accession *vrs1.a1* haplotype of AZ is AB489126 and *vrs1.a4* haplotype of SV169 and SV176 are AB478714.

Amplicon sequencing and the detection and analysis of variants. Genomic DNA of all 30 *agriocrithon* accessions was isolated from fresh leaves using an established protocol (Komatsuda *et al.*, 1998). The three amplicons generated were (1) a 2.1 Kbp fragment of *vrs1* including about 660 bp upstream of the 900 bp coding sequence and 510 bp downstream, (2) a 2.4 Kbp fragment of *btr1* including about 500 bp upstream of the 600 bp coding sequence and 1400 bp downstream, and (3) a 4.9 Kbp fragment of *btr2* including about 1900 bp upstream of the 600 bp coding sequence and 2400 bp downstream. Amplicons were generated, purified and sequenced as described previously (Komatsuda *et al.*, 2007, Pourkheirandish *et al.*, 2015). Sequencing reads were imported into Sequencher v4.8 (Gene Codes Corp., Ann Arbor, MI, USA) for assembly and low quality bases at the read ends were removed. Seventy-two haplotypes of *vrs1*, 81 haplotypes of *btr1* and 127 haplotypes of *btr2* present in ssp. *vulgare* and ssp. *spontaneum* were downloaded from GenBank (accession numbers provided in (Saisho *et al.*, 2009, Pourkheirandish *et al.*, 2015)).

Phylogenetic analysis. The sequences were aligned using the ClustalW algorithm implemented in the MEGA 6 package (www.megasoftware.net). Singleton variants (i.e., those detected in just one accession) were confirmed by manual checking of the sequencing trace. A phylogenetic analysis was carried out, using the neighbor-joining method in MEGA 6. Homogenous patterns among lineages and uniform rates of sites were assumed for the phylogenetic analysis. The pairwise-deletion option for treating gaps/missing data (Tamura *et al.*, 2011) was adopted. A bootstrap analysis (500 replicates) was performed to provide confidence estimates for the tree topology.

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Author contribution: M.P. assess row-type and rachis type. M.P., H.K., F.B., and J.W. carried out haplotype analysis. S.S. and T.K. performed allelism test. M.P. and T.K. designed the experiments. M.P., F.B., and T.K. wrote the manuscript. All authors have reviewed and commented on the manuscript.

Short Supporting Information Legends

Table S1. The 274 cultivars (H. vulgare ssp. vulgare) provided by the University of Okayama.

Table S2. The 150 wild barley (H. vulgare ssp. spontaneum) accessions provided by ICARDA.

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Figure legends

Figure 1. Schematic of the evolution of six-rowed barleys including the formation of *eu-agriocrithon*. In *eu-agriocrithon*, *vrs1.a1* is shared by HOR 4904, HOR 3886, HOR 3900, HOR 3552, HOR 8509 and HOR 7269, and *vrs1.a4* is shared by HOR 3904, HOR 3906, HOR 3905, HOR 3907, HOR 3902 and HOR 3903 (Table 1).

Figure 2. Allelism of *vrs1.a4* to the *vrs1* locus. (A) cv. Azumamugi (AZ, *vrs1.a1*). (B) Tilman Camp 1 (SV169, *vrs1.a4*). (C) Gho 1 (SV176, *vrs1.a4*). (D) F_1 resulting from hybridization of SV169 and AZ. (E) F_1 resulting from hybridization of SV176 and AZ. Both F_1 hybrids showed six-rowed spike confirming that *vrs1.a4* is an allele at *vrs1* locus. Scale bar = 1 cm.

Figure 3. Gene tree of the *vrs1* locus based on re-sequencing of 150 wild and 274 domesticated accessions (Saisho *et al*., 2009) and 30 *agriocrithon* accessions from this

study. Haplotype codes appear in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covers 2,067 nt and reveals 72 haplotypes. Haplotypes occurring in six-rowed accessions are shown in blue and split into four allele groups (*vrs1.a1* to *vrs1.a4*). Haplotypes occurring in two-rowed are shown in black except for Hap8 (*vrs1.a4*) present in both six-rowed (ssp. *vulgare* and *agriocrithon*) and two-rowed accessions (ssp. *spontaneum*) are in blue. Haplotypes existing in *agriocrithon* are marked with circles. Hap10 appears only in *deficiens* (rudimental lateral spikelets). Haplotypes appear in non-brittle are marked with asterisk except for Hap8 (*vrs1.a4*) appears in brittle rachis accessions too. Bootstrap values > 75% from 500 replicates are shown on the branches.

Figure 4. Gene tree of the *btr1* locus for the *Hordeum vulgare* germplasm panel with 150 wild and 274 domesticated accessions (Pourkheirandish *et al*., 2015) plus 30 *agriocrithon* accessions from this study. Abbreviated haplotype codes H000 stand for B1Hap000 in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covering 2,562 nt reveals 86 haplotypes. *btr1* carriers are shown in orange and *Btr1* in black. Haplotypes existing in *agriocrithon* accessions are marked with dots. Dashed lines encircle haplotypes occurring in tough-rachis accessions except for those that also appear in *pseudo-agriocrithon* accessions. Haplotype H023 occurs in wild barley in addition to domesticated and *agriocrithon* accessions.

Figure 5. Gene tree of the *btr2* locus for the *Hordeum vulgare* germplasm panel with 150 wild and 274 domesticated accessions (Pourkheirandish *et al*., 2015) plus 30 *agriocrithon* accessions from this study. Abbreviated haplotype codes H000 stand for

B2Hap000 in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covers 5,181 nt and reveals 135 haplotypes. *btr2* carriers are shown in green, *Btr2* in black. Haplotypes existing in *agriocrithon* accessions are marked with dots. Dashed lines encircle haplotypes occurring in tough-rachis types except for those marked in blue that also present in *pseudo-agriocrithon*.

Figure 6. Origin of *pseudo-agriocrithon.* (A) Hybridization between domesticated barleys followed by recombination between the *btr1* and *btr2* genes created the *pseudo-agriocrithon.* †: B2Hap267 and B2Hap268 derived from B2Hap224 through consequential nucleotides substitution in the 5' non-coding region. (B) Hybridization between wild and domesticated barley followed by recombination between the *btr1* and *btr2* genes created the *pseudo-agriocrithon.*

Table 1. Allelic constitution for 30 lines of H. vulgare ssp. agriocrithon provided by IPK.

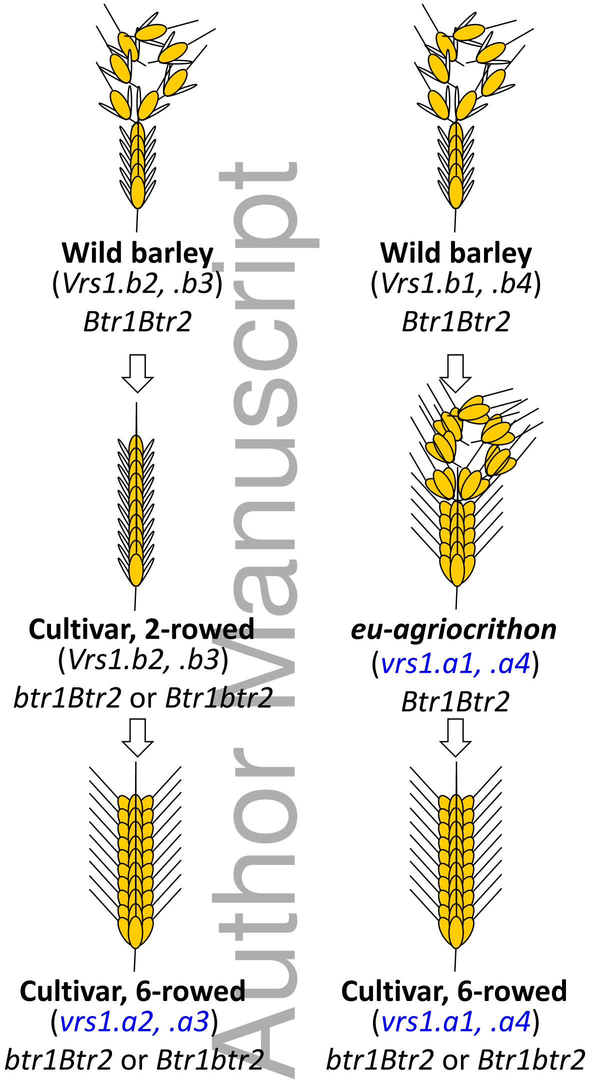

Figure 1. Schematic of the evolution of six-rowed barleys including the formation of *eu-agriocrithon*. In *eu-agriocrithon, vrs1.a1* is shared by HOR 4904, HOR 3886, HOR 3900, HOR 3552, HOR 8509 and HOR 7269, and *vrs1.a4* is shared by HOR 3904, HOR 3906, HOR 3905, HOR 3907, HOR 3902 and HOR 3903 (Table 1).

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Figure 2. Allelism of *vrs1.a4* to the *vrs1* locus. (A) cv. Azumamugi (AZ, *vrs1.a1*). (B) Tilman Camp 1 (SV169, *vrs1.a4*). (C) Gho 1 (SV176, *vrs1.a4*). (D) F_1 resulting from hybridization of SV169 and AZ. (E) F_1 resulting from hybridization of SV176 and AZ. Both F_1 hybrids showed six-rowed spike confirming that *vrs1.a4* is an allele at *vrs1* locus. Scale bar = 1 cm.

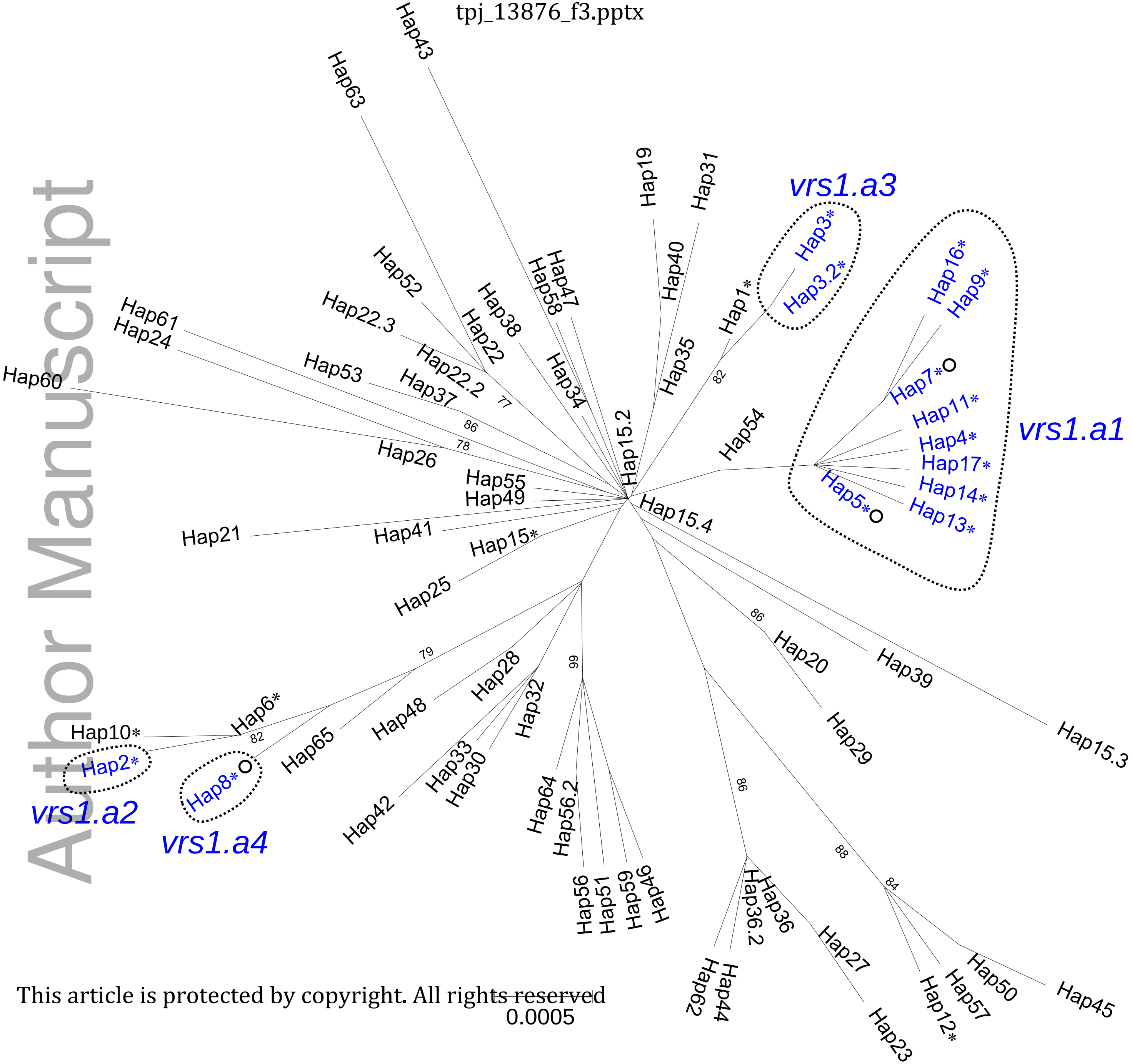
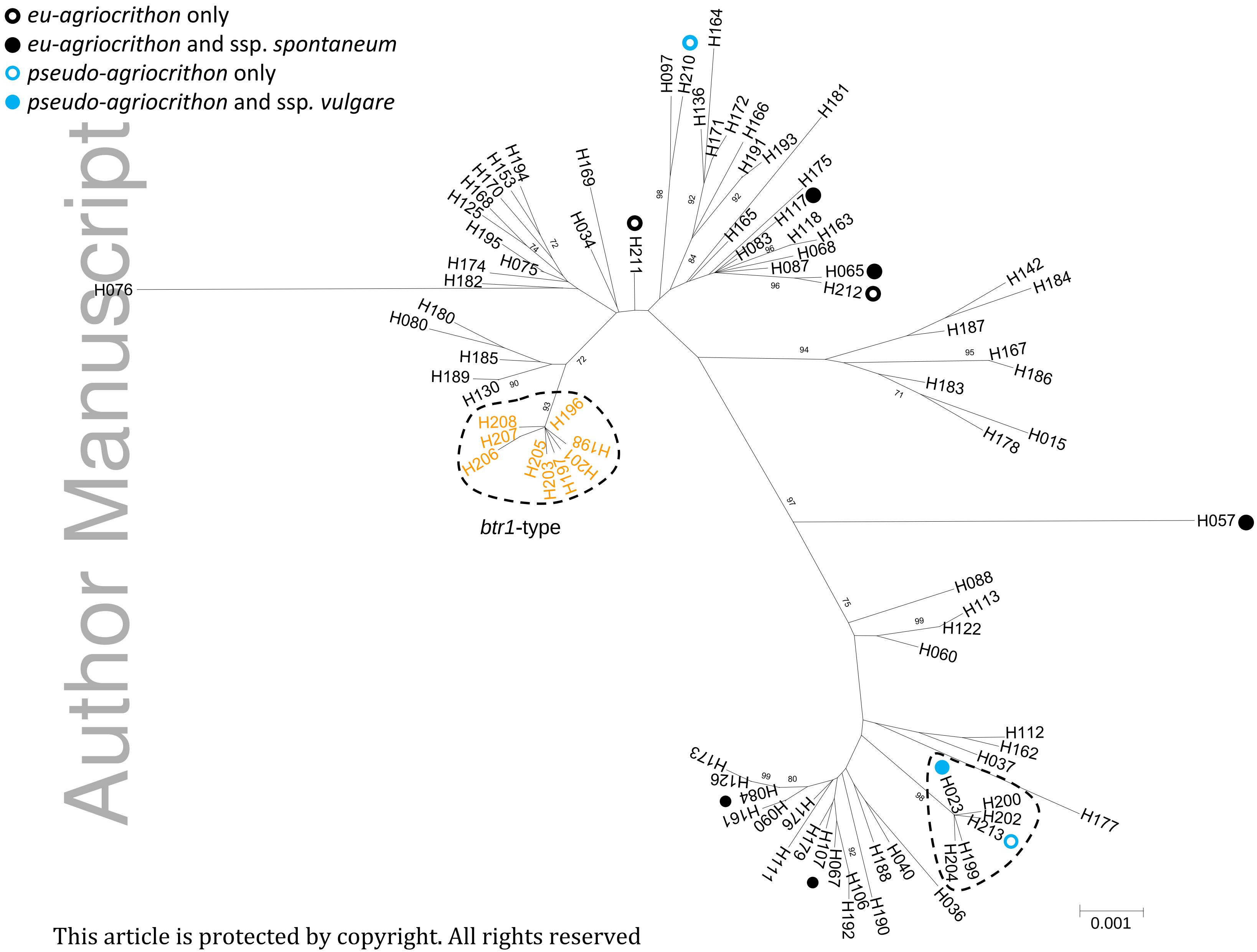

Hap43
Hap63
Hap19
Hap31
vrs1.a3
Hap3*
Hap3.2*
Hap1*
Hap40
Hap35
Hap47
Hap58
Hap52
Hap38
Hap22
Hap22.3
Hap22.2
Hap61
Hap24
Hap53
Hap37
Hap60
Hap34
Hap15.2
Hap54
vrs1.a1
Hap16*
Hap9*
Hap7*O
Hap11*
Hap4*
Hap17*
Hap14*
Hap13*
Hap5*O
82
77
86
78
Hap26
Hap55
Hap49
Hap21
Hap41
Hap15*
Hap15.4
Hap25
86
Hap20
Hap39
Hap29
Hap15.3
79
Hap6*
Hap10*
82
Hap2*
vrs1.a2
Hap8*O
vrs1.a4
Hap65
Hap48
Hap28
Hap32
99
Hap42
Hap33
Hap30
Hap64
Hap56.2
Hap56
Hap51
Hap59
Hap46
86
Hap62
Hap44
Hap36.2
Hap36
Hap27
Hap23
88
84
Hap12*
Hap57
Hap50
Hap45
0.0005

Figure 3.

Figure 3. Gene tree of the *vrs1* locus based on re-sequencing of 150 wild and 274 domesticated accessions (Saisho *et al*., 2009) and 30 *agriocrithon* accessions from this study. Haplotype codes appear in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covers 2,067 nt and reveals 72 haplotypes. Haplotypes occurring in six-rowed accessions are shown in blue and split into four allele groups (*vrs1.a1* to *vrs1.a4*). Haplotypes occurring in two-rowed are shown in black except for Hap8 (*vrs1.a4*) present in both six-rowed (ssp. *vulgare* and *agriocrithon*) and two-rowed accessions (ssp. *spontaneum*) are in blue. Haplotypes existing in *agriocrithon* are marked with circles. Hap10 appears only in *deficiens* (rudimental lateral spikelets). Haplotypes appear in non-brittle are marked with asterisk except for Hap8 (*vrs1.a4*) appears in brittle rachis accessions too. Bootstrap values > 75% from 500 replicates are shown on the branches.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● *eu-agriocrithon* and ssp. *spontaneum*
○ *pseudo-agriocrithon* only
● *pseudo-agriocrithon* and ssp. *vulgare*
H076
H097
H210
H164
H136
H171
H172
H166
H181
H191
H193
H175
H117
H165
H118
H163
H083
H068
H087
H065
H212
H211
H169
H034
H194
H153
H170
H168
H125
H195
H075
H174
H182
H180
H080
H185
H189
H130
H208
H207
H206
H196
H198
H201
H197
H203
H205
btr1-type
H142
H184
H187
H167
H186
H183
H178
H015
H057
H088
H113
H122
H060
H112
H162
H037
H177
H023
H200
H202
H213
H199
H204
H036
H173
H126
H084
H161
H090
H176
H179
H107
H111
H067
H106
H192
H190
H188
H040
0.001

Figure 4.

Figure 4. Gene tree of the *btr1* locus for the *Hordeum vulgare* germplasm panel with 150 wild and 274 domesticated accessions (Pourkheirandish *et al*., 2015) plus 30 *agriocrithon* accessions from this study. Abbreviated haplotype codes H000 stand for B1Hap000 in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covering 2,562 nt reveals 86 haplotypes. *btr1* carriers are shown in orange and *Btr1* in black. Haplotypes existing in *agriocrithon* accessions are marked with dots. Dashed lines encircle haplotypes occurring in tough-rachis accessions except for those that also appear in *pseudo-agriocrithon* accessions. Haplotype H023 occurs in wild barley in addition to domesticated and *agriocrithon* accessions.

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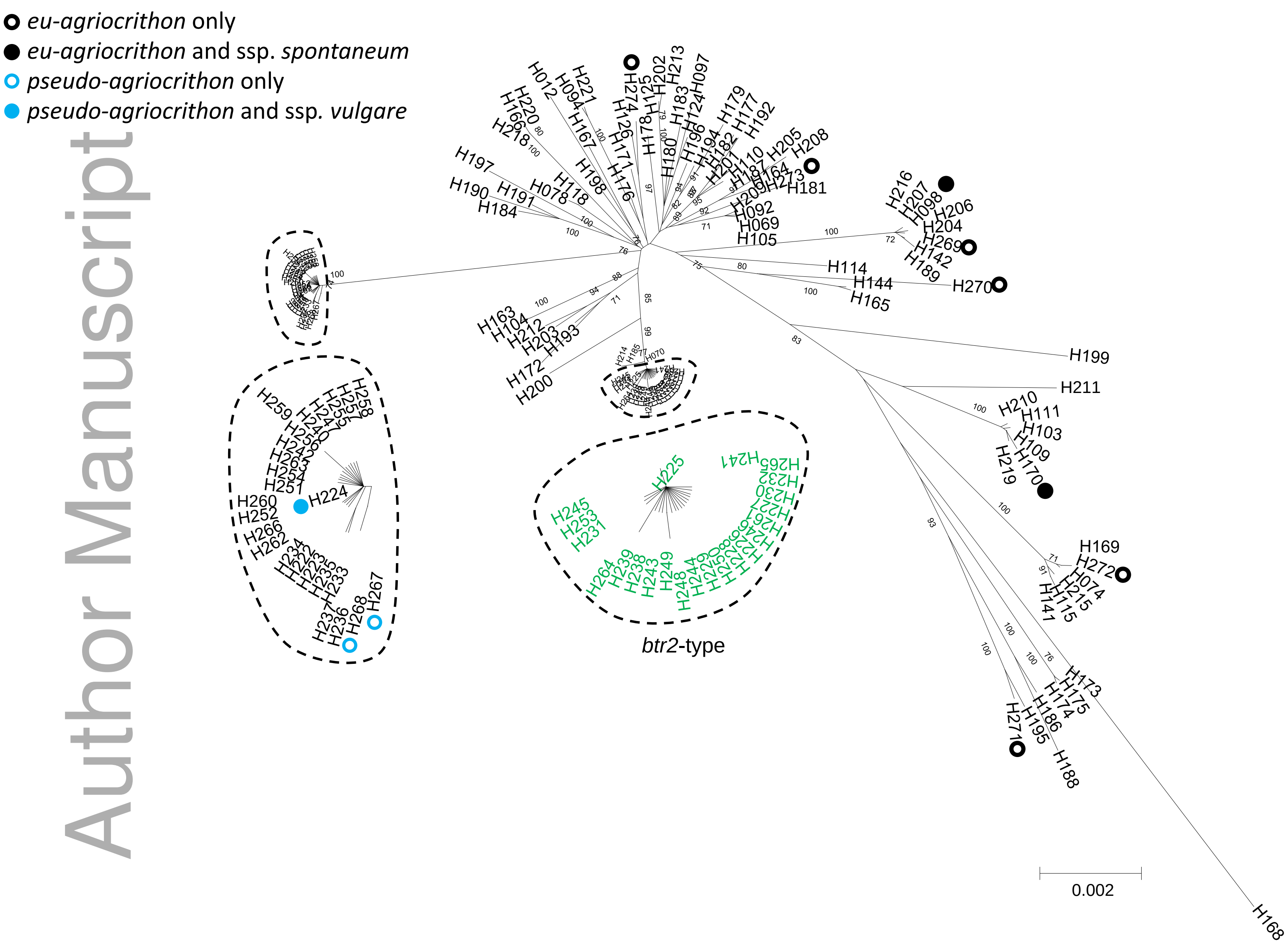
○ *eu-agriocrithon* only
● *eu-agriocrithon* and ssp. *spontaneum*
○ *pseudo-agriocrithon* only
● *pseudo-agriocrithon* and ssp. *vulgare*
H012
H221
H094
H220
H166
H218
H167
H274
H126
H171
H125
H202
H213
H178
H183
H097
H124
H180
H196
H179
H177
H194
H192
H182
H201
H110
H205
H208
H187
H164
H273
H181
H209
H092
H069
H105
H197
H190
H191
H184
H078
H118
H198
H176
H216
H207
H098
H206
H204
H269
H142
H189
H270
H114
H144
H165
H163
H104
H212
H203
H193
H172
H200
H214
H185
H070
H199
H211
H210
H111
H103
H109
H219
H170
H169
H272
H074
H215
H115
H141
H173
H175
H174
H186
H195
H271
H188
H168
H259
H258
H260
H224
H252
H266
H262
H234
H222
H223
H235
H233
H237
H236
H268
H267
H225
H265
H241
H232
H230
H245
H253
H231
H264
H239
H238
H243
H249
H248
H244
H229
btr2-type
0.002
100
80
100
97
76
75
88
94
71
85
66
83
93
72
91

Figure 5.

Figure 5. Gene tree of the *btr2* locus for the *Hordeum vulgare* germplasm panel with 150 wild and 274 domesticated accessions (Pourkheirandish *et al*., 2015) plus 30 *agriocrithon* accessions from this study. Abbreviated haplotype codes H000 stand for B2Hap000 in Table 1 and supplementary Tables 1 and 2. The multiple sequence alignment covers 5,181 nt and reveals 135 haplotypes. *btr2* carriers are shown in green, *Btr2* in black. Haplotypes existing in *agriocrithon* accessions are marked with dots. Dashed lines encircle haplotypes occurring in tough-rachis types except for those marked in blue that also present in *pseudo-agriocrithon*.

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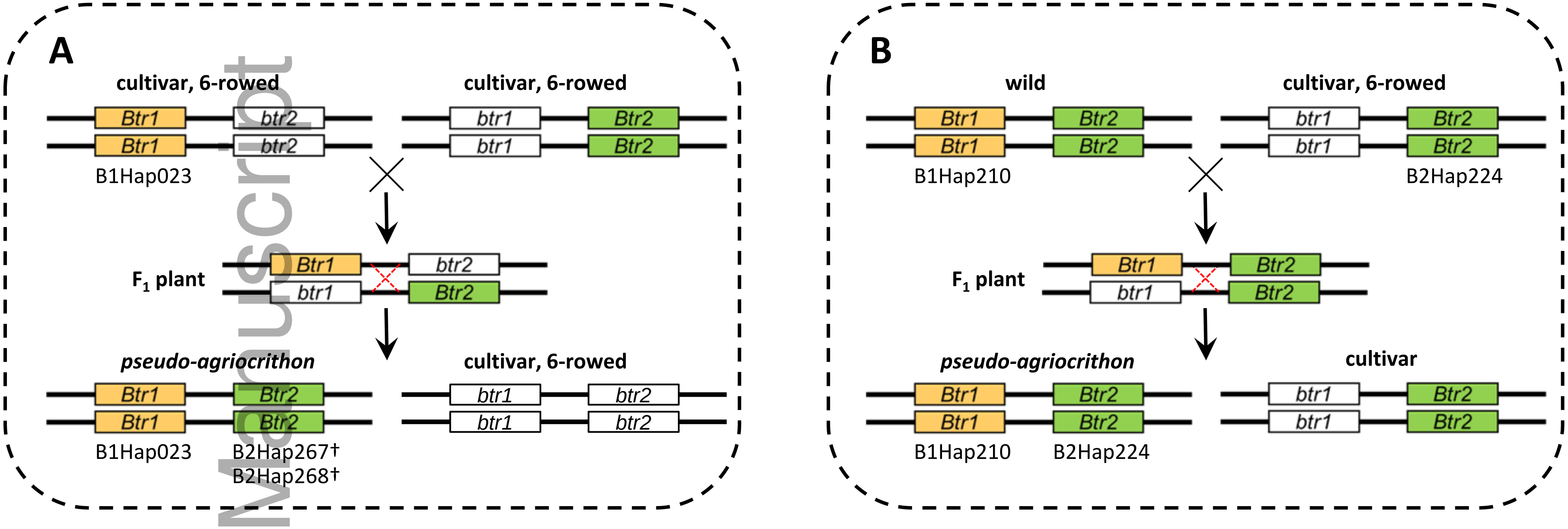

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