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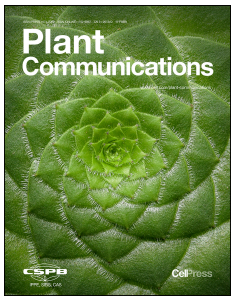

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Most Tibetan weedy barleys originated via recombination between *Btr1* and *Btr2* in domesticated barley

Running title: Origin of Tibetan weedy barleys

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Short summary: This study elucidates the origin and genetic characteristics of Tibetan weedy barleys. The brittle rachis and non-brittle rachis traits align with the haplotypes of the *btr1* and *btr2* genes, concluding that Tibetan weedy barleys originate via cross-pollinated hybridization of domesticated barley, followed by hybrids self-pollination and recombination between *Btr1* and *Btr2*.

Abstract

Tibetan weedy barleys reside at the edges of qingke (hulless barley) fields in Tibet. The spikes of these weedy barleys contain or lack a brittle rachis, with either two- or six-rowed spikes and either hulled or hulless grains at maturity. Although the brittle rachis trait of Tibetan weedy barleys is similar to that of wild barley (*Hordeum vulgare* ssp. *spontaneum* Thell.), these plants share genetic similarity with domesticated barley. The origin of Tibetan weedy barleys remains debated. Here, we show that most Tibetan weedy barleys originated from the cross-pollinated hybridization of domesticated

barleys, followed by hybrids self-pollination and recombination between *Non-brittle rachis 1* (*btr1*) and *2* (*btr2*). We discovered the specific genetic ancestry of these weedy barleys in South Asian accessions. Tibetan weedy barleys exhibit lower genetic diversity in comparison to those of wild and Chinese landraces/cultivars, and share a close relationship with qingke, genetically differing from the typical eastern and western barley populations. Tibetan weedy barleys were classified into two groups, brittle rachis (named BR) and non-brittle rachis (NBR); these traits align with the haplotypes of the *btr1* and *btr2* genes. Whereas wild barleys carry haplotype combinations of *Btr1* and *Btr2*, each showing lower proportions in a population, the recombinant haplotype BTR2H8+BTR1H24 is predominant in the BR group. Haplotype block analysis based on whole-genome sequencing revealed two recombination breakpoints, which are present in 80.6% and 16.8% of BR accessions using marker-assisted diagnosis. Hybridization events between wild and domesticated barley were rarely detected. These findings support the notion that Tibetan weedy barleys originated via recombination between *Btr1* and *Btr2* in domesticated barley.

Keywords: Tibetan weedy barley, *agriocrithon*, de-domestication, out-pollination, recombination, brittle rachis

Introduction

De-domestication is the process by which domesticated crops reacquire archaeological, ecological, or agronomic aspects of traits belonging to their wild relatives. Through this process, plants no longer rely on intensive human management and become self-sustaining and independently reproducing populations in nature (Wu et al., 2021). De-domestication, an important evolutionary phenomenon (Wu et al., 2021), is widely observed in domesticated plants such as rice (*Oryza sativa*; Ishikawa et al. 2005; Londo et al. 2007; He et al., 2017; Li et al., 2017; Qiu et al., 2017; Qiu et al., 2020), wheat (*Triticum aestivum*; Guo et al., 2020), barley (*Hordeum vulgare*; Konishi, 2001; Tanno and Takeda, 2004; Pourkheirandish et al., 2018; Guo et al., 2022), and eggplant (*Solanum melongena*; Page et al., 2019). Weedy rice (*O. sativa* f. *spontanea*), a de-domesticated form of domesticated rice, has undergone environmental adaptation and regained the grain-shattering character, allowing grains to disperse naturally in the field at maturity (Ishikawa et al. 2005; Londo et al. 2007; He et al., 2017; Li et al., 2017; Qiu et al., 2017; Qiu et al., 2020). Another example is Tibetan semi-wild wheat (*T. aestivum* ssp. *tibetanum* Shao), which exhibits genetic components similar to those of local wheat landraces, but the spike rachis turns brittle at maturity (Guo et al., 2020).

Wild barley (*Hordeum vulgare* ssp. *spontaneum* Thell.), originating from the Fertile Crescent, has spikes of brittle rachises and two-rowed kernels. The non-brittle rachis trait found in cultivated barley is controlled by either of two tightly linked gene loci, *Non-brittle rachis 1* (*btr1*) or *btr2* (Pourkheirandish et al., 2015), and the six-rowed spike trait is determined by the *Six-rowed spike 1* locus (*vrs1*; Komatsuda et al., 2007). To discriminate from two-rowed wild barley (ssp. *spontaneum*) in the Near East, the six-rowed barley with a brittle rachis collected by Åberg from Tibet (Åberg, 1938) was named "*agriocrithon"* (*H. vulgare* ssp. *agriocrithon* [Åberg] Bowd.). This plant has the brittle rachis trait characteristic of wild barley but produces six-rowed spikes. This observation prompted the hypothesis that Tibet might be an independent domestication center of cultivated barley (Åberg, 1938; Dai et al., 2012). However, this hypothesis was questioned based on a study using molecular markers for *btr1* and *btr2* (Pourkheirandish et al., 2018) and was not supported by studies involving genome-wide

sequencing (Lister et al., 2018; Zeng et al., 2018; Guo et al., 2022).

Three hypotheses have been proposed for the origin of *agriocrithon*: (1) a spontaneous mutation occurred at the *Vrs1* locus in *H. spontaneum*, resulting in six-rowed brittle spikes (Åberg, 1940; Schiemann, 1951); (2) the six-rowed *vrs1* allele was introgressed into wild barley, as it can naturally cross-pollinate with domesticated barley (Zohary, 1964; Konishi, 2001; Tanno and Takeda, 2004); and (3) de-domestication occurred via recombination of the separately originated *btr1Btr2* and *Btr1btr2* genotypes of domesticated barley, which would generate *Btr1Btr2* recombinants with functional alleles at both the *Btr1* and *Btr2* loci (Bothmer et al., 1995; Pourkheirandish et al., 2018). Based on the haplotype combinations between *Btr1* and *Btr2*, *agriocrithon* was classified into two groups, *eu-agriocrithon* and *pseudo-agriocrithon* (Pourkheirandish et al., 2018). *eu-agriocrithon*, which is mostly found in Central Asia, inherited the *Btr1Btr2* haplotypes from wild barley and was considered to have descended from multiple rounds of hybridization between diverse accessions of wild and domesticated barleys (Guo et al., 2022). *pseudo-agriocrithon* contains combined haplotypes that are exclusively present in domesticated barley at the *Btr1* and *Btr2* loci, implying that it originated from hybridization and recombination of six-rowed domesticated barleys that carry *btr1Btr2* and *Btr1btr2* (Pourkheirandish et al., 2018). However, these studies, involving functional genes analysis (Pourkheirandish et al., 2018) or population diversity analysis (Guo et al., 2022), relied on a limited number of *agriocrithon* accessions that have six-rowed brittle spikes, and didn't disclose the specific genetic ancestry of Tibetan weedy barleys.

Tibetan weedy barley refer ssp. *agriocrithon* and its intermediate barleys, which occur as weeds only at the edges of fields in Tibet (Zeng et al., 2018). It has been known as weeds by Tibetans for generations, and also has been described by some barley researchers as either Tibetan semiwild (Hsu, 1975; Ma et al., 1987; Ma, 1988) or Tibetan wild barley (Dai et al., 2012; Dai et al. 2014). It should be specified that Tibetan weedy barley is not an official name in standard barley taxonomy, and it is popularly used in order to identify qingke from other Tibetan barleys. The National Crop Genebank of China (NCGC) hosts >3000 Tibetan weedy barley accessions, along with

information about their morphologic variations in row type (two-rowed *vs.* six-rowed) and caryopsis trait (hulled *vs.* naked). This collection can be used to further decipher the population genomic variations in the accessions and to trace their genetic evolution. In this study, we investigated the population diversity of 965 barley accessions including 248 Tibetan weedy barleys using the Barley multiplex PCR amplification assay (BarPlex v1.0), a newly developed low-density genotyping assay. We conducted whole-genome sequencing (WGS) of 20 accessions, combined with analysis of previously published WGS datasets from 100 wild (WILD100) and 200 domesticated barleys (CORE200; Jayakodi et al., 2020), 98 qingke landraces/cultivars (Zeng et al., 2018), 11 Tibetan weedy barleys (Zeng et al. 2018), and 17 *eu-agriocrithon* accessions (Guo et al., 2022). We also re-sequenced the coding regions of *Btr1* and *Btr2* in 549 accessions and recorded the brittle or non-brittle rachis trait. Based on population genomics, functional gene diversity analysis, and marker-assisted analysis, we determined that Tibetan weedy barleys have evolved from domesticated ancestors via hybridization and recombination.

Results

Tibetan weedy barleys share high genetic similarity with qingke

The BarPlex v1.0 assay is a complexity-reduced genotyping tool that targets 500 unique fragments distributed genome-wide, with 54 to 84 fragments on each chromosome (Supplemental Table 1 and Supplemental Figure 1A). We conducted independent experiments in which we analyzed the 965 accessions in six sub-populations including 248 Tibetan weedy barleys, 51 wild barleys, 191 qingke landraces/cultivars, 214 Chinese landraces (without qingke), 234 Chinese cultivars (without qingke), and 27 exotic lines (Figure 1A, 1B, Table 1, and Supplemental Table 2). The average and median detection rates of the 500 target fragments in each accession were 99.7% and 100%, respectively (Supplemental Figure 1B and 1C). For each of the 965 accessions, the mean and median detection rates were 99.7% and 99.8%, respectively (Supplemental Figure 1D), with an average sequencing depth of 735 across accessions (Supplemental Figure 1E).

We applied two matrices to analyze the diversity across sub-populations: (1) target SNPs, i.e., the 500 expected SNPs (one SNP on each fragment), and (2) multiple SNPs (mSNPs), i.e., all polymorphisms in the captured fragments. We did not observe a significant difference in the number or size of the captured fragments across sub-populations (Table 1). Fewer polymorphisms in the targeted SNP sites and mSNP sites were observed in qingke and Tibetan weedy barley than in Chinese landraces and cultivars. Wild barley also showed fewer target SNPs than the other sub-populations, except for qingke, which exhibited a similar number of target SNPs to wild barley (Table 1). This is probably due to the sources of the target SNPs, which were identified from domesticated barley; thus, a number of these SNPs were monomorphic in wild barley. By exploring the mSNPs that were derived from the 116.5-kb sequences of captured fragments, we observed a higher number of SNPs in wild barley, but not in Tibetan weedy barley, than in the other sub-populations (Table 1).

Wild barley showed the highest nucleotide diversity (π) among sub-populations, whereas both Tibetan weedy barley and qingke showed low intra-population diversity (Figure 1C). The smallest genetic differentiation was between Tibetan weedy barley and qingke (*Fst* = 0.0810), while both sub-populations exhibited greater differences from the other sub-populations (Figure 1C). We performed a maximum likelihood estimation of ancestral genetic components using ADMIXTURE, with the number of ancestral populations (K) ranging from 4 to 5 (Figure 2A). At K = 4, the genetic composition of Tibetan weedy barley was nearly identical to that of qingke, whereas at K = 5, a specific component (shown in lime green) was found in Tibetan weedy barleys, but not in the other sub-populations. This finding corresponds to the discrete clusters obtained by principal component analysis (PCA; Figure 2B) and neighbor-joining (NJ) phylogenetic analysis (Figure 2C). The Tibetan weedy barley and qingke sub-populations were present within the same cluster, away from the wild barley and Chinese landraces/cultivars (Figure 2B and 2C). Except for a few accessions with admixture compositions between clades, each of the sub-populations was clearly separated (Figure 2). These results reveal a close genetic relationship between Tibetan weedy barley and qingke.

The specific genetic ancestry identified in Tibetan weedy barleys was detected in South Asian accessions

We traced the specific genetic components of Tibetan weedy barley in the barley gene pools by performing ADMIXTURE analysis based on the integrated variation matrices of BarPlex v1.0 and those of WILD100 and CORE1000, globally representative diversity panels for the wild and domesticated barley gene pools, respectively (Milner et al., 2019). At $K = 9$, the specific ancestral lineages (lime green) of Tibetan weedy barley could be found in landraces collected from South Asian countries such as Pakistan, India, or Afghanistan (Supplemental Figure 2). In addition, we conducted whole-genome sequencing (WGS) of 20 Tibetan weedy barleys that showed diversified genetic compositions (Supplemental Figure 3). We performed population diversity analysis using these 20 samples and 11 Tibetan weedy barleys (Zeng et al., 2018), as well as previously published WGS datasets (Supplemental Table 3). Again, PCA and NJ phylogenetic analysis based on WGS markers clustered Tibetan wild barleys with qingke, with both techniques revealing their very close genetic relationship (Figure 3A and 3B). The cluster containing Tibetan wild barley and qingke includes 34 landraces and cultivars. At $K = 3$, the specific ancestral lineages (lime green) of Tibetan weedy barley were found in accessions from Pakistan, India, Nepal, and Afghanistan (Figure 3C and 3D). Collectively, the specific genetic components found in Tibetan weedy barleys were identified in South Asian accessions.

Tibetan weedy barleys with the non-brittle rachis trait were identified

Unlike *eu-agriocrithon* and *pseudo-agriocrithon*, which are six-rowed barleys with the brittle rachis trait (Pourkheirandish et al. 2018), the Tibetan weedy barleys hosted by the NCGC show variations in terms of row type and caryopsis adhesiveness. Of the 248 Tibetan weedy barley accessions examined, 102, 58, 54, and 34 contained six-rowed/hulled (TWB6H), six-rowed/naked (TWB6N), two-rowed/hulled (TWB2H), and two-rowed/naked (TWB2N) spikes, respectively (Supplemental Table 2). Among these, 159 accessions exhibited the brittle rachis trait, as expected (named BR), and the 89 remaining accessions showed the non-brittle rachis trait (NBR) (Supplemental Figure 4A and 4B). An overall low nucleotide diversity based on π was observed in

each of the four morphological variant sub-groups (TWB6H, TWB6N, TWB2H, and TWB2N; Supplemental Figure 4C, 4D, and 4E). A low degree of genetic differentiation (*Fst*) was detected among the four morphological sub-groups of Tibetan weedy barley. The six-rowed naked barleys (both BR and NBR barleys) showed the lowest *Fst* values compared to qingke.

The brittle rachis phenotype corresponds to the haplotype combinations *Btr1*/*Btr2*

Two tightly linked genes, *Btr1* and *Btr2*, are essential determinants of the brittle rachis trait in barley (Pourkheirandish et al., 2015). The combination of functional alleles at both *Btr1* and *Btr2* is required for the production of brittle rachises, whereas loss-of-function variants at either *btr1* (−1 bp) or *btr2* (−11 bp) lead to the non-brittle rachises. To uncover the genetic basis of the brittle rachis trait in Tibetan weedy barley, we sequenced the complete coding sequences (CDSs) of both genes in 248 Tibetan weedy barleys, together with 51 wild barleys, 60 qingke landraces/cultivars, 92 Chinese landraces, 71 Chinese cultivars, and 27 exotic lines.

For the 591-bp CDS of *Btr1*, we identified 12 haplotypes across the 549 sequenced accessions (Supplemental Table 4); all these haplotypes were reported previously (Pourkheirandish et al., 2015). Nine haplotypes were exclusively found in wild barley, and three (BTR1H18, BTR1H24, and BTR1H27) were shared among wild barley, Tibetan weedy barley, and/or domesticated barley (Figure 4A). Three Tibetan weedy barleys (Figure 5A) and one Chinese landrace (Supplemental Figure 5A) remained heterozygous (BTR1H18/24) at *Btr1*. In Tibetan weedy barley, two functional *Btr1* haplotypes (BTR1H24 and BTR1H27) and one loss-of-function *btr1* haplotype (BTR1H18; 1-bp deletion) were revealed, and BTR1H24 was a major haplotype, as it was present in 238 of the 245 homozygous accessions (97.1%; Figure 5A). All five accessions that carried BTR1H18 (encoding pre-mature protein) had the non-brittle rachis trait. For wild barley, we identified three accessions from IPK Genebank that carried the non-functional BTR1H18 and showed a non-brittle rachis as well (Supplemental Figure 6).

For the 692-bp CDS of *Btr2*, we identified 13 *Btr2* haplotypes across the sequenced accessions, including two loss-of-function *btr2* haplotypes (Figure 4B; Supplemental

Table 5). These haplotypes were described previously (Pourkheirandish et al., 2015). Eight accessions, including two wild barleys, five Tibetan weedy barleys, and one Chinese barley landrace, remained heterozygous at *Btr2* (either BTR2H1/8 or BTR2H8/24; Figure 5A; Supplemental Figure 5 and 6). Their progeny showed the expected 3:1 segregation ratio (3 brittle *vs.* 1 non-brittle), as observed in segregating populations of the two Tibetan weedy barleys HA00064 and HA00095 (Supplemental Table 6). Among the 243 Tibetan weedy barleys (five accessions with heterozygous alleles were not included; Figure 5A), the two major haplotypes, BTR2H8 (functional *Btr2*) and BTR2H1 (non-functional *btr2*), were present in 64.6% (157/243) and 34.6% (84/243) of the population, respectively. Two functional haplotypes were rarely present: BRT2H9 (1/243) and BRT2H24 (1/243; Figure 5A). In wild barley, one accession from IPK Genebank carried a non-functional *btr2* (BTR2H1) and showed a non-brittle rachis spike (Supplemental Figure 6).

We then analyzed the haplotype combinations between the *Btr1* and *Btr2* loci (accessions with heterozygous loci were included). Of the 21 haplotype combinations identified from 51 wild barley accessions, 17 were homozygous for both functional alleles (*Btr1Btr2*), two were homozygous for functional *Btr1* but heterozygous at *Btr2*, and two haplotypes (*btr1Btr2* or *Btr1btr2*) were identified in four accessions (Supplemental Figure 6). The combination BTR2H1+BTR1H24 was predominant in Chinese landraces and qingke (Supplemental Figure 5A and 5B), while a high proportion of BTR2H8+BTR1H18 was found in Chinese cultivars and exotic lines as well (Supplemental Figure 5C and 5D). In Tibetan weedy barleys, three and two haplotype combinations (only referring to homozygotes) were found in BR and NBR lines, respectively. The haplotype combinations BTR2H8+BTR1H24 and BTR2H1+BTR1H24, which were predominant in domesticated barleys, were present in 98.7% (151/153) and 94.4% (84/89) of BR and NBR lines, respectively (Figure 5A). Overall, all accessions with functional *Btr1*/*Btr2* combinations showed the brittle rachis trait, whereas the non-brittle rachis trait was associated with either *btr1*/*Btr2* or *Btr1*/*btr2*.

Two recombination breakpoints were identified in Tibetan weedy barleys

The combination of heterozygous BTR2H1/8+BTR1H24 or BTR2H8+BTR1H18/24 was found in four Tibetan weedy barley accessions. These lines could generate BTR2H1+BTR1H24, BTR2H8+BTR1H24, or BTR2H8+BTR1H18 homozygotes, which were found in Tibetan weedy barleys (Figure 5A). Therefore, we hypothesized that the rachis brittleness of Tibetan weedy barleys originated from hybridization between domesticated barleys harboring *Btr1btr2* and *btr1Btr2*, followed by recombination between *Btr1* and *Btr2*. To test the hypothesis, we analyzed the WGS datasets of 20 Tibetan weedy barleys that showed diversified genetic compositions (Figure 5B) with previously published WGS data for 426 accessions, including 17 *eu-agriocrithon*, 11 Tibetan weedy barley, 100 wild barleys, 200 domesticated barleys, and 98 qingke accessions (Zeng et al., 2018; Jayakodi et al., 2020; Guo et al., 2022). We observed two recombination breakpoints. Breakpoint 1 occurred around 39,608,364 on chromosome 3H, and breakpoint 2 occurred around 39,695,117 on chromosome 3H. The two breakpoints are located within the physical interval containing the *Btr1* and *Btr2* genes in the vicinity of these genes (Figure 5C) and are identical to those observed in *pseudo-agriocrithon* (Guo et al., 2022). Specifically, for three accessions that had the functional *Btr1* haplotype and remained heterozygous at *Btr2*, all contained breakpoint 1, and they all generate brittle and non-brittle segregants (Supplemental Table 2). These results demonstrate that the brittle type BTR2H8+BTR1H24 emerged as a result of recombination following hybridization between lines carrying BTR2H8+BTR1H18 and BTR2H1+BTR1H24.

We did not detect breakpoints in two singleton haplotype combinations (BTR2H9+BTR1H27 and BTR2H24+BTR1H27) corresponding to two accessions with the brittle rachis trait (Figure 5C). These haplotypes were not found in domesticated barley (Supplemental Figure 5) but were detected in wild barley (Supplemental Figure 6), implying that these genotypes arose from crop–wild hybrids. Two other accessions (carrying BTR2H1/8+BTR1H18/24) were detected with admixed compositions and heterozygous loci beyond the interval of *Btr1*/*Btr2* (Figure 5B and 5C), implying that cross-pollination occurred between the domesticated barleys.

We developed diagnostic markers that targeted the two recombination breakpoints

and the functional variation sites at *btr1* (−1 bp) and *btr2* (−11 bp; Figure 6A). Of the 155 BR accessions examined (excluding two heterozygotes and two wild–crop hybrids), 129 and 26 accessions carried breakpoint 1 and breakpoint 2, respectively (Figure 6B). Within the 129 accessions carrying recombinant breakpoint 1, one and three remained heterozygous at the *Btr1* or *Btr2* locus, respectively. No recombination was detected in the NBR accessions (Figure 6B).

In summary, these results provide compelling evidence that Tibetan weedy barley arose due to natural hybridization between domesticated barleys, followed by two independent recombination events.

Discussion

Most Tibetan weedy barleys originated via two recombination events between *Btr1* and *Btr2*

Based on evidence from population genomics, functional gene diversity analysis, and haplotype block analysis, we conclude that most Tibetan weedy barleys were derived from two independent recombination events between *Btr1* and *Btr2* following the natural hybridization of domesticated barley. Our conclusion agrees with the findings of Pourkheirandish et al. (2018) and Guo et al. (2022), who applied gene-based haplotype analysis and genome-wide markers in their studies, respectively, to demonstrate that barleys with the brittle rachis trait found in Tibet resulted from the hybridization and recombination of domesticated barley. In addition to identifying the two critical recombination breakpoints, which are consistent with what has been reported before (Guo et al., 2022), we identified three accessions that remained heterozygous at the first breakpoint close to *Btr2*, providing direct evidence that the recombination event occurred within the *Btr1*/*Btr2* interval. Through self-pollinations, these lines might have produced both brittle and non-brittle descendants. De-domestication has triggered widespread interest in the resurgence of wild traits in plants within natural environments. These studies represent a novel mechanism of crop de-domestication through hybridization and recombination within domesticated species to obtain weediness, differing from *de novo* mutation or introgression that were reported

317 in weedy rice (Ishikawa et al., 2005; Londo et al., 2007; He et al., 2017; Li et al., 2017;
318 Qiu et al., 2020).

319 The two previously reported cases of the double recessive haplotype *btr1btr2* in
320 domesticated barley aligned with the recombination hypothesis (Guo et al., 2022). The
321 barleys with the brittle rachis trait examined in the study might have predominantly
322 resulted from two recombination events, as 155 of the 159 brittle rachis accessions (two
323 heterozygotes and two wild–crop hybrids are included) showed one or the other of them.
324 Since these *Btr1* and *Btr2* haplotype combinations were the same as those in
325 domesticated barley, they fit the description *pseudo-agriocrithon* (Pourkheirandish et
326 al. 2018). In this study, two Tibetan weedy barley accessions (HA00083, six-rowed;
327 HA00196, two-rowed) maintained heterozygosity (*Btr1btr1*/*Btr2btr2*), and both carried
328 the same haplotypes (BTR2H1/8+BTR1H18/24), which were exclusively present in
329 domesticated barley. These accessions are probably hybrids resulting from cross-
330 pollination and are theoretically able to produce new types of brittle rachis lines through
331 self-pollination once recombination has occurred. Notably, the recombination events
332 between the *Btr1* and *Btr2* loci would be extremely rare, since in barley gene pools only
333 two recombination events were identified, and in the segregation population comprising
334 28,116 F_2 plants, only four recombinants were identified. Therefore, we would like to
335 conclude that most Tibetan weedy barleys originated via two recombination events
336 between *Btr1* and *Btr2* in domesticated barley, although the recombination events were
337 rare.

338 Although Tibetan weedy barleys didn't play crucially in barely domestication, they
339 might be of interest in the study of the adaptative potential of bi-direction crop-wild
340 gene flow (Guo et al., 2022). They have evolved and adapted under harsh environments
341 (e.g. infertile, extreme cold, hypoxia) in Tibet, and future investigations might unlock
342 novel insights in the studies of fertility use efficiency, cold tolerance and seed
343 emergence under lower temperature condition.

344 **Wild–domesticated hybrids were identified within Tibetan weedy barleys**

345 *eu-agriocrithon* accessions carry the *Btr1* and *Btr2* haplotypes, which are extensively
346 present in wild barley, whereas *pseudo-agriocrithon* refers to the barley accessions that

carry either the *Btr1* or *Btr2* haplotype from domesticated barley (Pourkheirandish et al., 2018). While both *eu-agriocrithon* and *pseudo-agriocrithon* produce six-rowed spikes with brittle rachises, only *pseudo-agriocrithon* was reported to be present in Tibet (Pourkheirandish et al., 2018; Guo et al., 2022). However, in this study, we identified two accessions (HA00097, two-rowed, BTR2H9+BTR1H27; HA00098, two-rowed, BTR2H24+BTR1H27) carrying the *Btr1* and *Btr2* haplotypes that were exclusively carried in wild barley. These accessions did not show recombination at the *Btr1*/*Btr2* locus or an admixed genetic composition, as identified previously (Guo et al., 2022). This finding also agrees with results for *eu-agriocrithon* (Pourkheirandish et al., 2018) and supports the de-domestication scenario via hybridization between wild and domesticated barley (Konishi, 2001; Tanno and Takeda, 2004). However, this observation needs to be taken with caution, since it is currently unknown whether these two accessions descended from wild–domesticated hybrids that arose in Central Asia, followed by movement to Tibet, or whether more recent hybridizations occurred between wild and domesticated accessions in Tibet.

The specific genetic ancestry of Tibetan weedy barley traces back to accessions from South Asia

Tibetan weedy barleys, regardless of whether they had the BR or NBR trait, exhibited a very close genetic relationship with qingke, with admixed ancestries contributed by eastern barley (e.g. Chinese landraces), western barley (e.g., exotic lines), and wild barley (minor contribution; $K = 4$; Figure 2A). These barleys also carry a specific genetic ancestry (lime green) that was not found in any other sub-populations except qingke ($K = 5$; Figure 2A). Further analysis uncovered their specific genetic ancestry, which is similar to that of barley accessions from India, Pakistan, Nepal, and Afghanistan (Figure 3D). This discovery supports one of the presumed routes of qingke origination, in which their ancestor might have migrated through Afghanistan, Pakistan, India, and Nepal before reaching the southern Tibetan Plateau (Zeng et al., 2018). We observed diversification in terms of row type (two-rowed or six-rowed), caryopsis adhesiveness (hulled or naked), and seed shattering (brittle or non-brittle rachis) in Tibetan weedy barley. This finding on morphological diversity contradicts the notion

that the genetic diversity of Tibetan weedy barley is much lower than that of other lineages. High levels of ultraviolet (UV) radiation in high-altitude regions increase the frequency of cross-pollination events, leading to higher heterozygosity in self-pollinating crops (Llorens et al., 2015). The genetic diversity detected in Tibetan weedy barley based on morphological traits was higher than that observed based on sparser marker datasets (Konishi, 2001; Tanno and Takeda, 2004; Guo et al., 2022). This higher morphological diversity is thought to be due to the existence of numerous lines as well as genes that have maintained higher heterozygosity.

Methods

Plant materials and phenotyping

A total of 965 accessions were examined in this study, including 51 wild barleys, 248 Tibetan weedy barleys, 191 qingke landraces/cultivars (hereafter referred to as "qingke"), 214 Chinese landraces (CL, without qingke), 234 Chinese cultivars (CC, without qingke), and 27 exotic lines (EL; Supplemental Table 2). Two segregating populations derived from selfing of Tibetan weedy barley accessions HA00064 and HA00095 were analyzed as well. Two-week-old plants were transferred to a vernalization chamber for 35 days of incubation (4°C, 10-h-light/14-h-dark cycle), followed by cultivation under normal glasshouse conditions (22°C, 14-h-light/18°C, 10-h-dark cycle) until full maturity. Spike brittleness was determined as previously described (Pourkheirandish et al., 2015).

DNA extraction and quantification

Seedlings at the two-leaf stage were harvested for DNA extraction as previously described (Shi et al., 2019). The DNA quality was checked by agarose gel electrophoresis, and DNA quantity was assessed using a Qubit 3.0 fluorometer (Thermo Fisher, USA).

Barley multiplex PCR amplification assay (BarPlex v1.0) development: target SNP selection, primer design, PCR amplification, library construction, high-throughput sequencing, and bioinformatics analysis

Target SNPs were selected based on three sources of data: (1) SNPs revealed from

whole-genome sequencing of 14 landraces/cultivars (Supplemental Table 7), (2) genotyping-by-sequencing (GBS)-derived polymorphisms in a global barley diversity panel (Milner et al., 2019), and (3) SNPs included in a 50K Illumina Infinium iSelect array (Bayer et al., 2017). Flanking sequences of the target SNPs were extracted from the barley reference genome (MorexV3; Mascher et al., 2021) and subjected to primer selection using BatchPrimer 3 (You et al., 2008).

The first round of PCR amplification using a GenoPlexs multiple PCR amplification kit (MolBreeding, China) was performed in a reaction volume of 30 μL, containing 10 ng genomic DNA as template, 10 μL GenoPlexs Master Mix (3×, including high-fidelity polymorphism and PCR buffers), and equal molar amounts of mixed primers. The PCR cycling conditions included denaturation at 95°C for 5 min, followed by six cycles of 30 sec at 95°C and then 4 min at 60 °C and a final extension at 72°C for 5 min. The PCR products were purified by adding 15 μL GenoPrep DNA Clean Beads solution (MolBreeding, China), followed by two rounds of washing with 75% ethanol. The purified PCR products were used as templates for the second round of amplification in which 1 μL of barcode solution and 10 μL of 3× GenoPlexs Master Mix were added to the reaction, and the same PCR cycling conditions described above were employed. PCR products from the second round were purified in the same manner and eluted with 30 μL Tris-HCl solution (pH 8). The specificity and fragment sizes of the target products were checked by agarose gel electrophoresis, and the quantity was measured using a Qubit 3.0 fluorometer (Thermo Fisher, USA). Equal molar amounts of barcoded PCR products from different barley accessions were mixed and sequenced in PE150 mode on the DNBSEQ-T7 platform (MGI, Shenzhen, China). In total, 587 primer pairs were tested for multiplex PCR followed by high-throughput sequencing, and 87 pairs were discarded due to a lower capacity for fragment capture, the capture of multiple fragments, or too high/low PCR amplification efficiency.

The raw reads were filtered using fastp v0.20.0 (Chen et al., 2018) with the parameters "−q = 20, −u = 40, and −n = 10" to remove low-quality reads and adapter sequences. Clean reads were mapped to the barley reference genome (MorexV3; Mascher et al., 2021) using BWA-MEM v0.7.17 (Li and Durbin, 2009). SNP calling

was performed using the *UnifiedGenotype* function of GATK v3.5.0 (McKenna et al., 2010) with the parameters "-dcov 1000000, -minIndelFrac 0.15, -glm BOTH, and -l INFO", followed by a filtration using the *VariantFiltration* function with the parameters "MQ0 ≥ 4 && (MQ0 / (1.0 * DP) > 0.1) and DP < 5 || QD < 2". SNPs/INDELs with allele frequency (AF) ≥ 0.8 or AF ≤ 0.2 were considered to be homozygous, and those with 0.2 < AF < 0.8 were considered to be heterozygous.

Whole-genome sequencing (WGS)

Twenty Tibetan weedy barley accessions were subjected to WGS (average ~52.5 Gb, 12.4×). Genomic DNAs were used for library construction and sequenced in PE150 mode on the DNBSEQ-T7 platform (BGI, Shenzhen, China). Published WGS datasets from materials including 100 wild (WILD100) and 200 domesticated barleys (CORE200; Jayakodi et al., 2020), 98 qingke landraces/cultivars (Zeng et al., 2018), 11 Tibetan weedy barleys (Zeng et al. 2018), and 17 *eu-agriocrithon* accessions (Guo et al., 2022) were included in this study. Filtering of raw reads, alignment to the reference genome, variant detection, and variation filtration were performed as previously described (Guo et al., 2022; Huang et al., 2023).

Population diversity analysis

WGS variant call format (vcf) files of WILD100 and CORE1000 (Milner et al., 2019) were merged with the variation matrices of BarPlex v1.0, and the intersection was extracted using Bcftools v1.10.2 (Li, 2011). Population structure analysis was performed using ADMIXTURE v1.3.0 (Alexander et al., 2009) with 10 different random seeds at each *K*. The Q proportions were averaged across the 10 replications with CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007). Principal component analysis (PCA) was performed with PLINK v1.9 (Purcell et al., 2007). A neighbor-joining (NJ) tree was constructed based on the pairwise genetic distances using TreeBeST v.1.9.2 (Varella et al., 2019) with 1000 bootstrap replicates. Nucleotide diversity (π) was calculated using DNASP v6.12.01 (Librado and Rozas, 2009). Differentiation index (*Fst*) was calculated using VCFtools v0.1.16 (Danecek et al., 2011) with a window size of 10 bp and a window step of 10 bp. The haplotype block diagram was generated using the NGenomeSyn program (https://github.com/hewm2008/NGenomeSyn).

Sanger sequencing, Kompetitive allele-specific PCR (KASP) genotyping, and haplotype network analysis

PCR products were amplified, purified, and sequenced as described previously (Pourkheirandish et al., 2015) using newly developed primers (Supplemental Table 8). Kompetitive allele-specific PCR (KASP) primers targeting the non-functional alleles at *Btr1* and *Btr2* were developed (Supplemental Table 8) and used for genotyping as described (Shi et al., 2019). Sequence variation was analyzed using Sequencher v4.8 (Gene Codes Corp., https://www.genecodes.com/). Two hundred and eight haplotypes of *Btr1* and 263 haplotypes of *Btr2*, which were previously reported (Pourkheirandish et al., 2015), were downloaded from NCBI GenBank and included in the haplotype diversity analysis in conjugation with the sequences obtained from this study. Median-joining (MJ) networks of the haplotypes were constructed using PopART v1.7 (Leigh et al., 2015).

Author Contribution Statement

P.Y., M.M., J.Z., J.L. and Z.F. designed the research; G.G., L.Y., Y.C. and S.T. performed the experiments; G.G., P.Y., Y.G. and Q.H. analyzed data; P.Y., G.G., T.K., and C.J. wrote the manuscript.

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Data availability statement

The short reads generated by high-throughput Illumina sequencing have been deposited in the public NCBI database (PRJNA758510, PRJNA758513, PRJNA758514, and PRJNA758516). The accession IDs for each entry are listed in Supplemental Table 2.

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Table and figure legends

Figure 1. Geographic distribution and genetic diversity of the 965 barley accessions. (A) The collection sites based on passport information for the accessions (Supplemental Table 2). **(B)** Snapshot of the geographic distribution of Tibetan weedy barley (TWB) and qingke accessions. **(C)** Nucleotide diversity (π) and population divergence (*Fst*) across the five sub-populations. The value in each circle represents π within the sub-population, and the value on each line indicates *Fst* between sub-populations.

Figure 2. Population diversity of 965 geographically referenced barley accessions.

647 **(A)** The ADMIXTURE ancestry coefficients (K = 4, 5) for sub-populations comprising
648 Tibetan weedy barley (TWB; NBR: non-brittle rachis; BR: brittle rachis), wild barley,
649 qingke landraces/cultivars, Chinese cultivars (CC, without qingke), Chinese landraces
650 (CL, without qingke), and exotic lines (EL). The length of each segment in each vertical
651 bar represents the proportion contributed by ancestral populations. **(B)** Principal
652 component analysis (PCA) of 965 accessions based on 3133 multiple SNPs (mSNPs).
653 **(C)** Neighbor-joining (NJ) phylogenetic tree based on genetic distance. In **(B)** and **(C)**,
654 green, purple, orange, blue, red, and brown represent TWB, wild barley, qingke, CL,
655 CC, and EL, respectively.
656 **Figure 3. Population diversity of 446 WGS accessions.** This collection included 20
657 Tibetan weedy barleys from this study and 426 samples comprising WILD100 and
658 CORE200 (Jayakodi et al. 2020), 98 qingke and 11 Tibetan weedy barleys (Zeng et al.
659 2018), and 17 *eu-agriocrithon* accessions (Guo et al., 2022). **(A)** Principal component
660 analysis. **(B)** Neighbor-joining (NJ) phylogenetic tree based on genetic distance. **(C)**
661 ADMIXTURE ancestry coefficients (K = 2 and 3) for 157 accessions. **(D)** Twenty-five
662 landraces/cultivars from South Asia exhibited the specific genetic component (K = 3;
663 lime green) found in Tibetan weedy barleys. PAK, Pakistan; NPL, Nepal; IND, India;
664 AFG, Afghanistan; NA, Unknown.
665 **Figure 4. Median-joining (MJ) networks for the *Btr1* and *Btr2* haplotypes. (A)** MJ
666 network of the *btr1* locus for the *Hordeum vulgare* germplasm panel containing 43
667 haplotypes from 208 wild (gray) and domesticated (beige) accessions (Pourkheirandish
668 et al., 2015) plus 545 samples from this study. Multiple sequence alignment covering
669 591 nucleotides revealed 43 haplotypes (Supplemental Table 4). **(B)** MJ network of the
670 *btr2* locus for the *H. vulgare* germplasm panel with 45 haplotypes from 263 wild (gray)
671 and domesticated (beige) accessions (Pourkheirandish et al., 2015) plus 541 samples
672 from this study. Multiple sequence alignment covering 693 nucleotides revealed 45
673 haplotypes (Supplemental Table 5). The accessions with heterozygous genotypes at this
674 locus were not included. Wild barley, Tibetan weedy barley (TWB), qingke, Chinese
675 cultivars (CC), Chinese landraces (CL), and exotic lines (EL) are indicated in purple,
676 light/dark green, orange, red, blue, and brown, respectively. Light green, TWB (BR);

dark green, TWB (NBR).

Figure 5. The patterns of recombination between the *Btr1* and *Btr2* genes in Tibetan weedy barley. (A) Types of recombination in the 248 Tibetan weedy barleys. The *Btr2* and *Btr1* haplotypes and their combinations are shown. **(B)** Types of haplotype recombination in 20 WGS Tibetan weedy barley accessions. **(C)** Haplotype block analysis around *Btr1* and *Btr2* (chr3H: 39.5–39.8 Mb in MorexV3). The colored circles on the left indicate the haplotype combination of each accession based on the color legend in (B). *The variant information was adapted from Guo et al. (2022). BR: brittle rachis, NBR: non-brittle rachis.

Figure 6. Validation of the recombination breakpoints in Tibetan weedy barley using diagnostic Kompetitive allele-specific PCR (KASP) markers. (A) Genotyping with four KASP markers. The blue, red and green dots represent the allele specfici fluorescence signal FAM/FAM (reference homozygote), VIC/VIC (alternative homozygote) and FAM/VIC (heterozygote), respectively. NTC: no template control (orange). **(B)** Statistics of the genotyping results from 244 Tibetan weedy barleys. Two accessions that remained heterozygous at both the *Btr1* and *Btr2* loci and two Tibetan weedy barley accessions that appeared to be crop–wild accession hybrids were not included. *The accessions remained heterozygous at either *Btr1* or *Btr2*.

Supplemental Figure 1. PCR-based target amplification (BarPlex v1.0) of 500 genomic fragments. (A) Chromosomal locations of targeted fragments in the reference genome (v3) of barley cultivar 'Morex'. **(B)** Percentage of the detection rate of all fragments (detected accessions divided by 965 at each fragment). **(C)** The log2 value of the sequencing depth in each fragment. **(D)** Percentage of the detection rate of all accessions (detected fragments divided by 500 in each accession). **(E)** The log2 value of the sequencing depth in each accession. Error bars represent standard deviation (SD).

Supplemental Figure 2. ADMIXTURE ancestry coefficients (*K* = 7 to 9) based on 3101 multiple SNPs (mSNPs). (A) 1532 accessions that correspond to four collections comprising Tibetan weedy barley, qingke landraces/cultivars, WILD100, and CORE1000 (Milner et al., 2019). The colored blocks below the bar plots correspond to the geographical groupings in **(B)**. The gray block corresponds to North American

accessions that are not shown on the map. NBR: non-brittle rachis; BR: brittle rachis. **(B)** The color code for geographical origins as formerly described (Milner et al., 2019). **(C)** Twenty landraces from South Asia exhibited the specific genetic component (K = 9; lime green) found in Tibetan weedy barley. PAK, Pakistan; IND, India; AFG, Afghanistan.

Supplemental Figure 3. The 20 Tibetan weedy barley accessions subjected to whole-genome sequencing.

Supplemental Figure 4. Nucleotide diversity (π) and population divergence (*Fst*) across the sub-groups of Tibetan weedy barley and qingke groups. Photographs of the non-brittle (left) and brittle rachis (right) trait in two-rowed **(A)** and six-rowed **(B)** Tibetan weedy barley. Nucleotide diversity (π) and population divergence (*Fst*) in different morphological groups within the 248 Tibetan weedy barleys **(C)**, the 159 Tibetan weedy barleys with the brittle rachis trait **(D)**, and the 89 Tibetan weedy barleys with non-brittle rachis trait **(E)**. The value in each circle represents a measure of nucleotide diversity (π) for this group, and the value on each line indicates population divergence (*Fst*) between the two groups. TWB2H: Tibetan weedy barley/two-rowed/hulled; TWB2N: Tibetan weedy barley/two-rowed/naked; TWB6H: Tibetan weedy barley/six-rowed/hulled; TWB6N: Tibetan weedy barley/six-rowed/naked.

Supplemental Figure 5. The combination of *Btr1* and *Btr2* haplotypes in 92 Chinese landraces (CL; A), 60 qingke landraces/cultivar (Qingke; B), 71 Chinese cultivars (CC; C), and 27 exotic lines (EL; D).

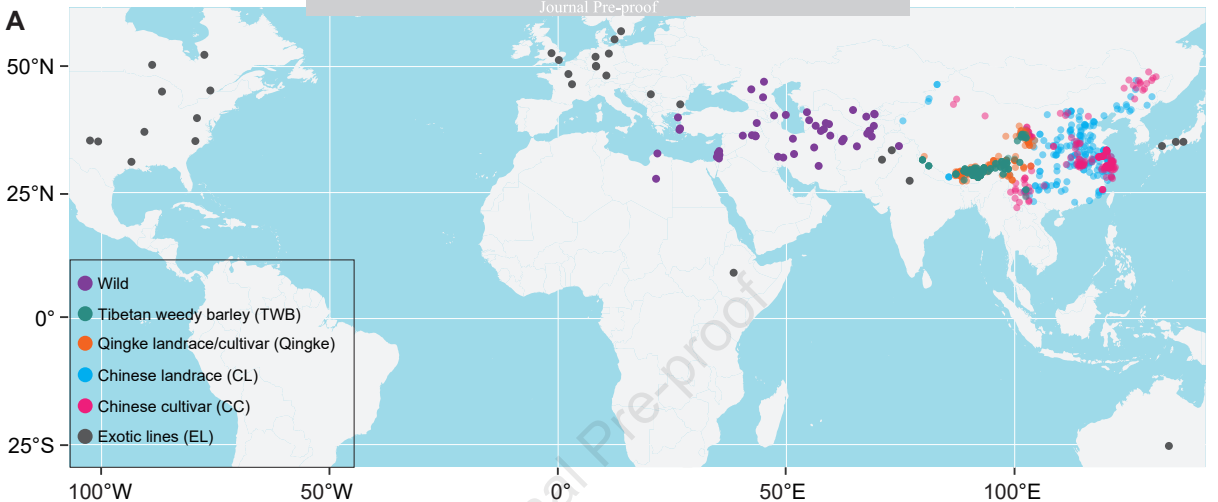
Supplemental Figure 6. The combination of *Btr1* and *Btr2* haplotypes in 51 wild barleys.

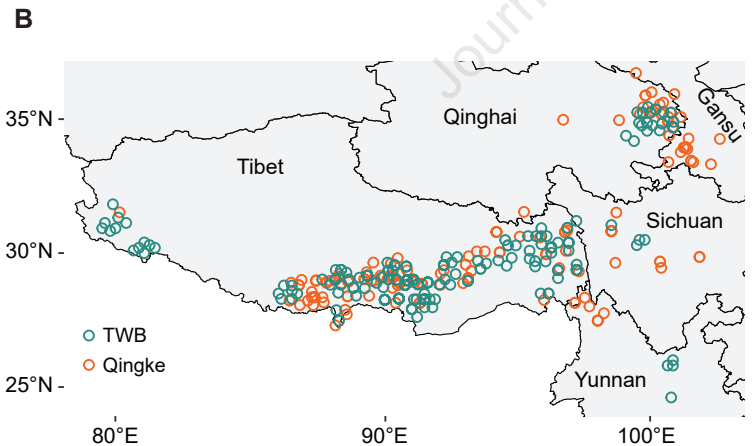
Table 1 Information about the 500 captured fragments, SNPs, and multiple SNPs (mSNPs) detected in 965 accessions

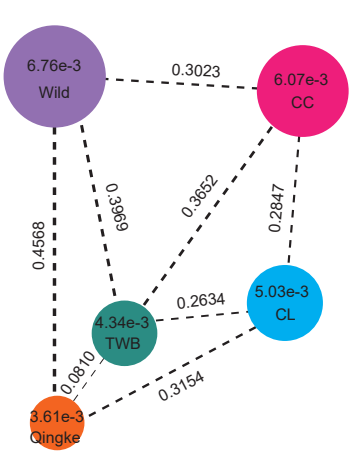
Parameters	Wild accessions	Tibetan weedy barleys	Qingke	Chinese landraces	Chinese cultivars	Exotic lines
Number of accessions	51	248	191	214	234	27
Detected target fragments	500	500	500	500	500	500

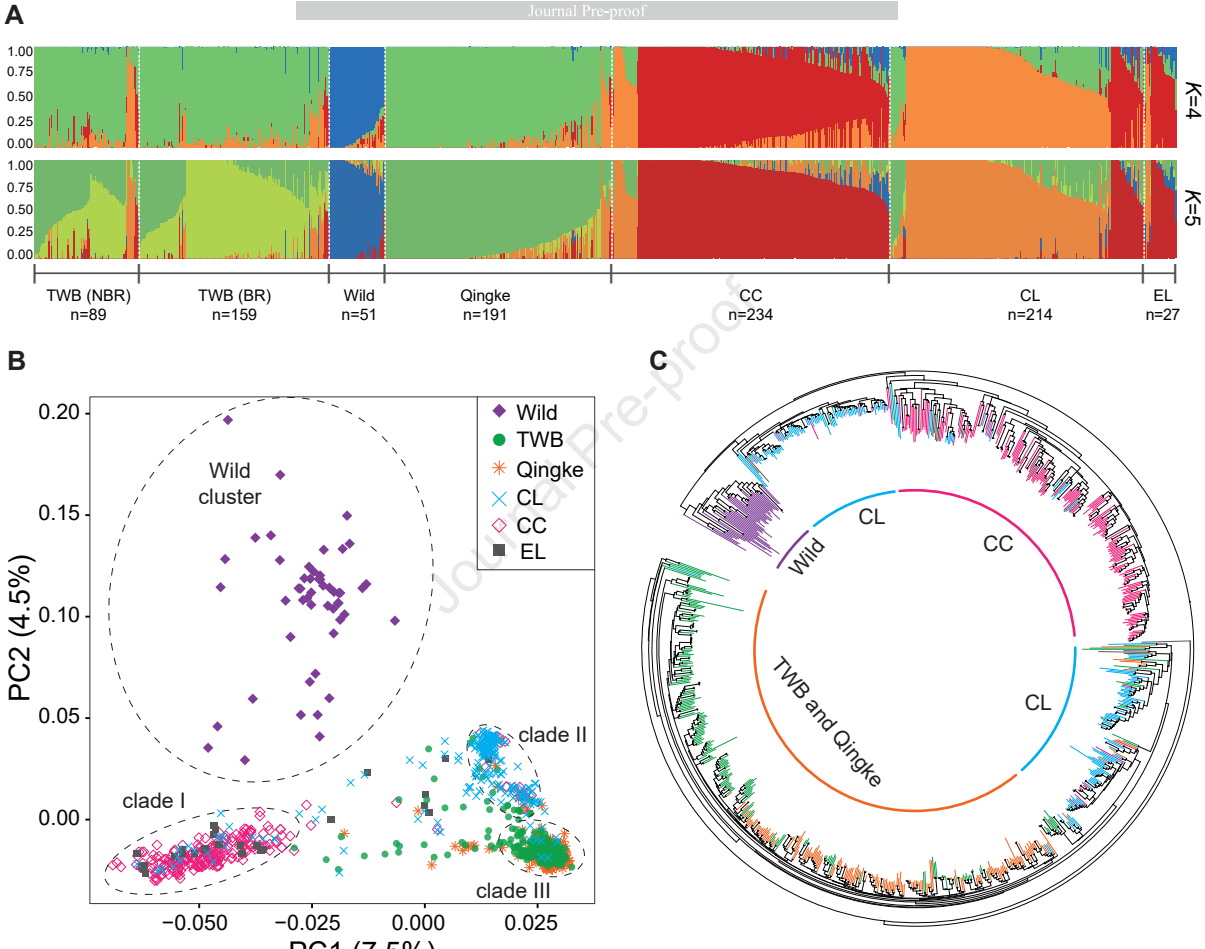
Fragment size (kb)[1]	116.5	116.5	116.5	116.5	116.5	116.5
Average depth (×)	508	458	883	800	867	989
Polymorphic target SNPs	306	351	304	382	392	379
Polymorphic mSNPs	2,620	1,475	1,254	1,601	1,497	1,344
Nucleotide diversity (π)	6.76e-3	4.34e-3	3.61e-3	5.03e-3	6.07e-3	6.88e-3

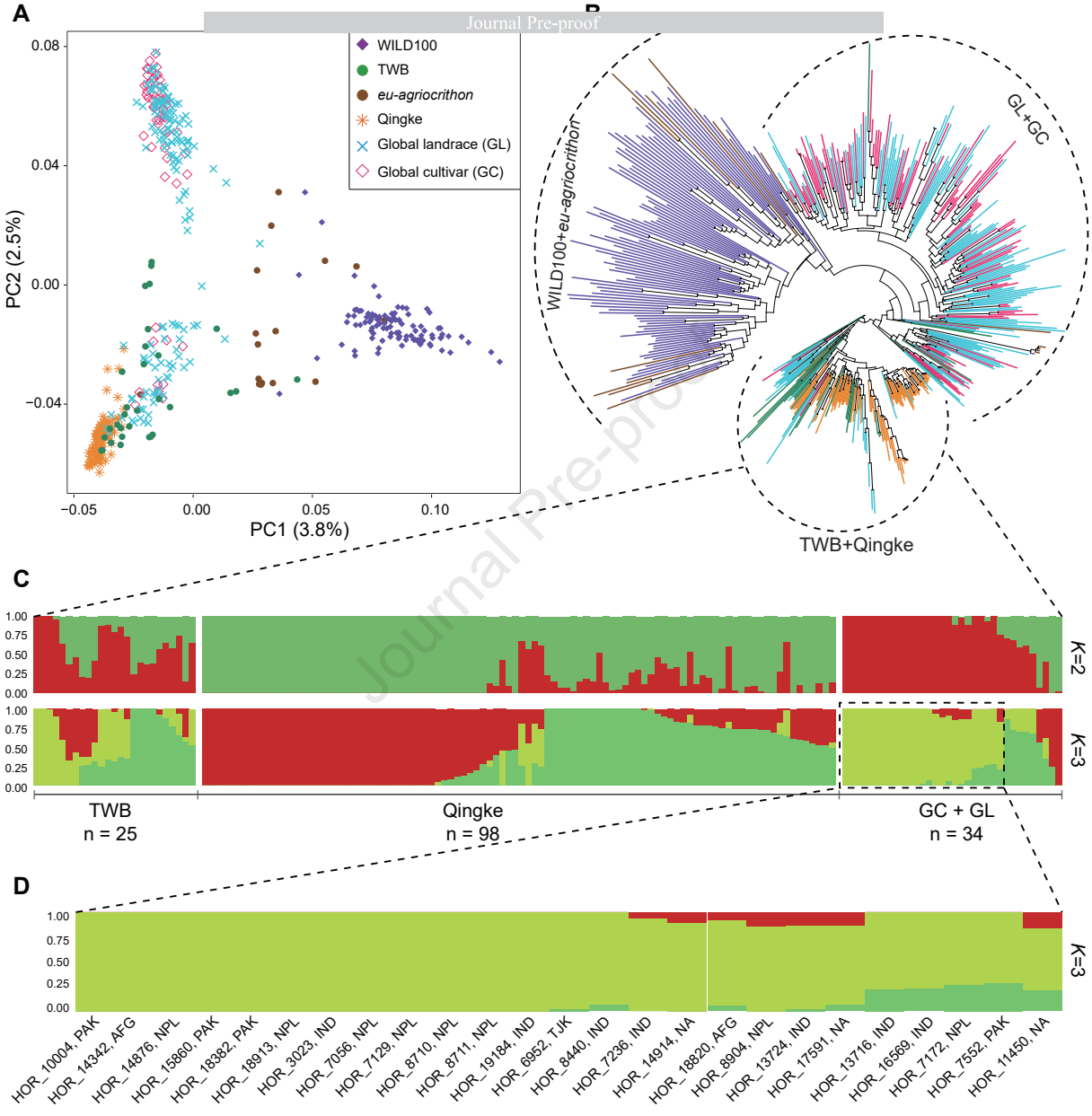
733 [1] Fragment size was calculated based on the barley reference genome (MorexV3; Mascher et al. 2021).

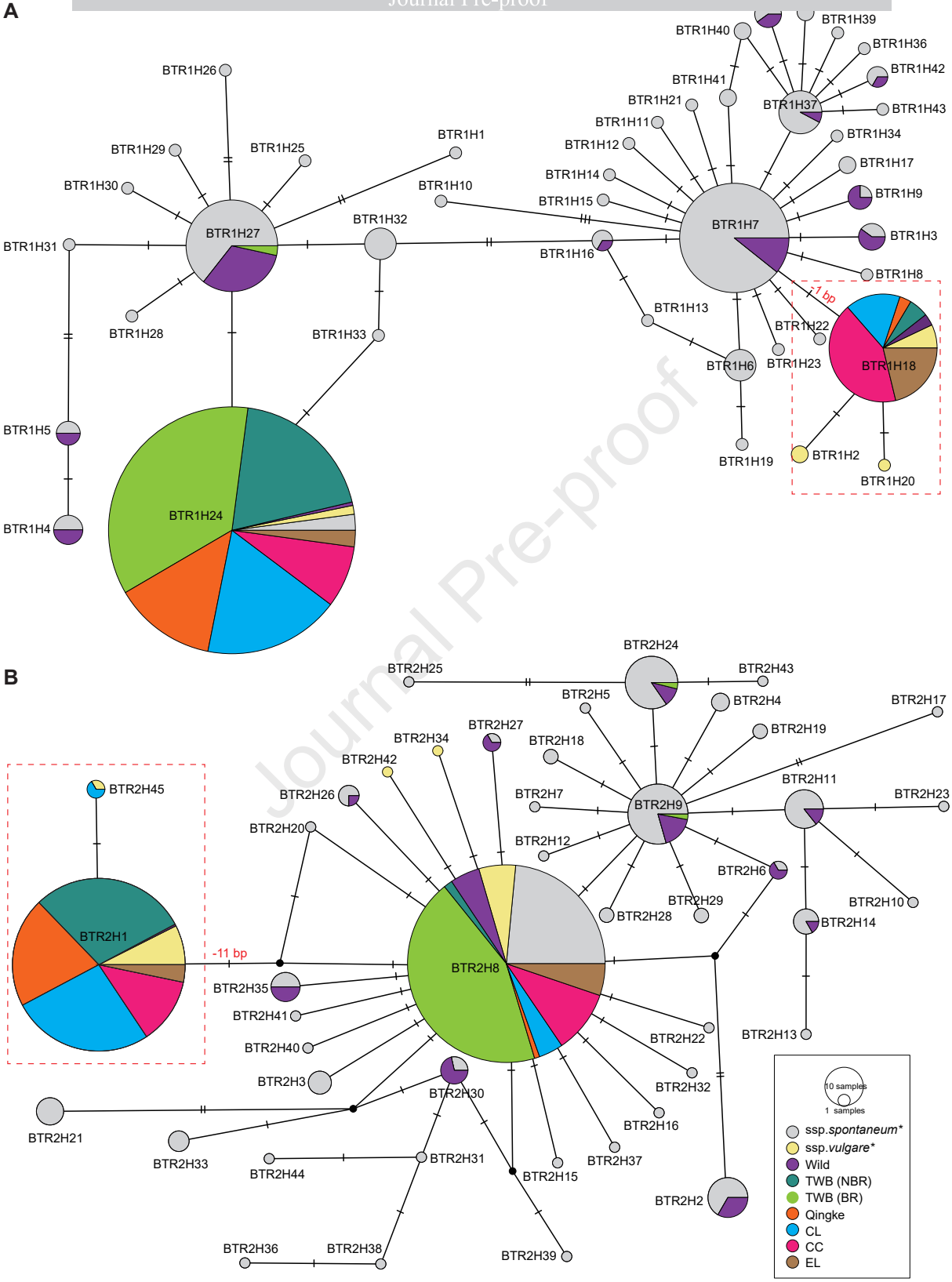


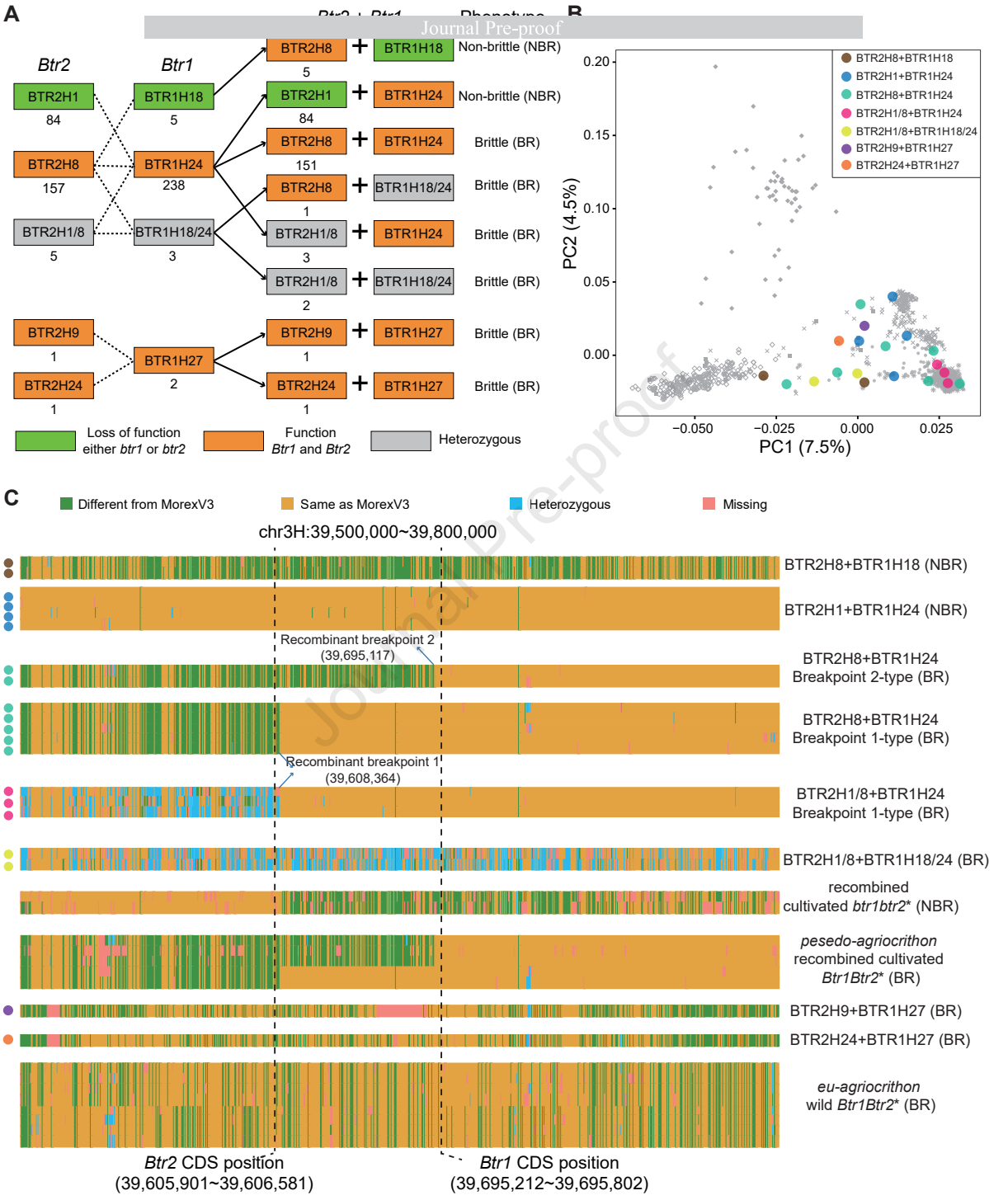












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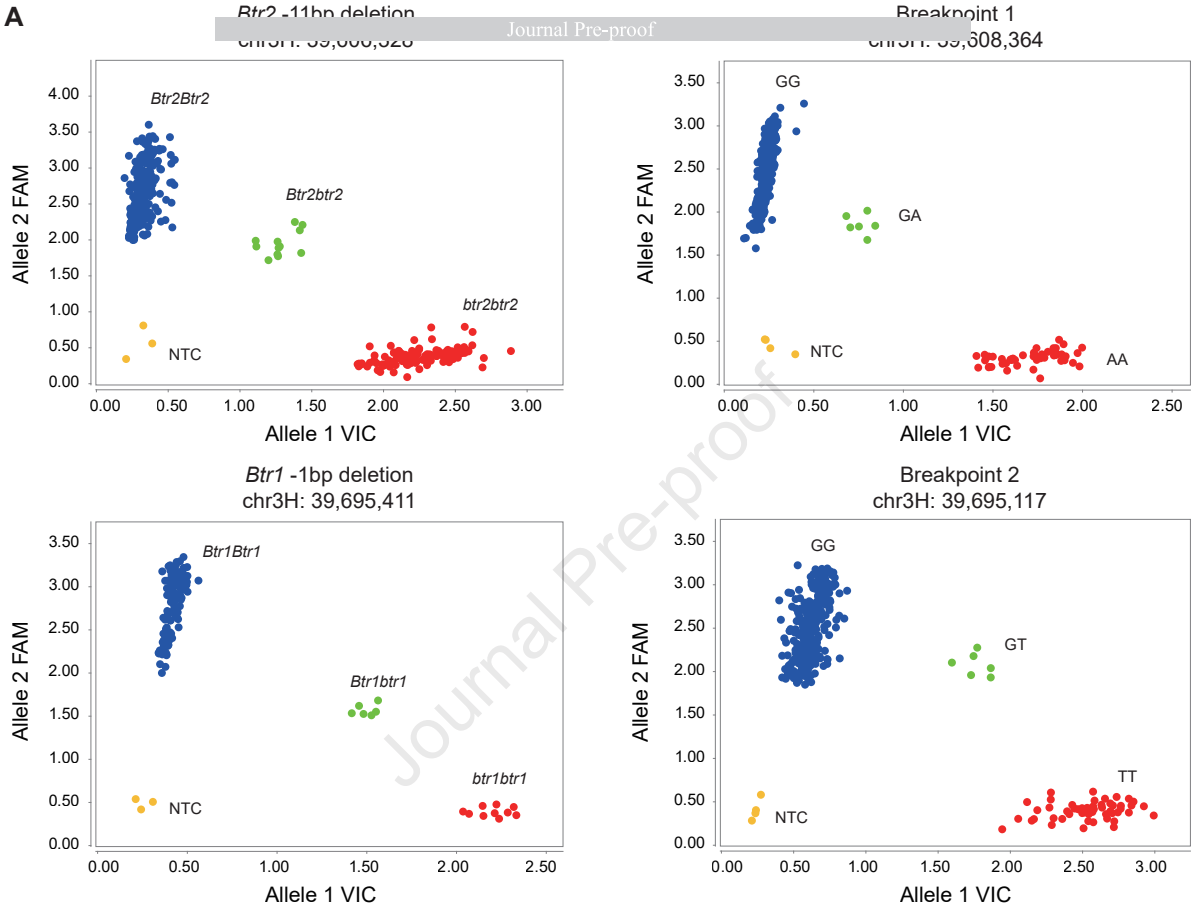

B

Btr2 genotype	*Btr1* genotype	Recombination Type	Phenotype	Number	Rate %
Btr2Btr2	*btr1btr1*	No-recombination	Non-brittle	5	-
btr2btr2	*Btr1Btr1*	No-recombination	Non-brittle	84	-
Btr2Btr2	*Btr1Btr1*	Breakpoint 1-type	Brittle	125	80.6
Btr2Btr2	*Btr1Btr1*	Breakpoint 2-type	Brittle	26	16.8
Btr2btr2	*Btr1Btr1*	Breakpoint 1-type	Brittle	3*	1.9
Btr2Btr2	*Btr1btr1*	Breakpoint 1-type	Brittle	1*	0.7
Total				244	-