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Evaluation of Barley—Introgression Lines Between Annual Barley and the Perennial *Hordeum bulbosum* in a Cold-Temperate Climate

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Abstract

Perennial wild relatives of annual crops possess traits associated with stress tolerance, efficient nutrient uptake, and increased soil carbon sequestration. They therefore represent valuable genetic resources for developing perennial crops that may reduce environmental and climate impacts and enhance food security. We evaluated 151 introgression lines (ILs) derived from crosses between barley and the perennial relative *Hordeum bulbosum*, each carrying unique *H. bulbosum* chromosome segments in a barley genetic background. Growth and regrowth after harvest were evaluated in two field trials, established by spring and fall planting in separate years in central Sweden, and under simulated two-year seasonal cycles in a climate chamber. In the climate chamber, 54% of the ILs exhibited strong regrowth, and two-thirds of these ILs showed reproductive growth across two seasonal cycles. In the field, most ILs showed regrowth after harvest but did not survive the cold winter. The parental genetic background had a strong effect from the barley parent on several growth and reproductive traits, but some ILs exhibited phenotypes not expected based on the barley parent. Of the divergent ILs with high regrowth, identified by PCA combining all studied traits, *H. bulbosum* introgressions at chromosome arms 2HL and 4HL were highly represented among ILs with cultivar Emir as a barley parent, and introgressions at 1HL among ILs with cultivar Morex as a parent. Perennial growth was observed only under mild conditions, indicating that perenniality of these ILs in a cold-temperate climate requires not only regrowth capacity but also adaptation to photoperiod, vernalization, and frost. ILs with high regrowth and reproductive growth may be used in crossing efforts with cold-tolerant germplasm to support the development of perennial barley.

Keywords: *Hordeum bulbosum*; genetic resources; introgression line; perennial; regrowth; wild relatives

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1. Introduction

Annual cereals are among the most widely cultivated crops worldwide and form the foundation of the human diet. To achieve continuous increases in productivity, annual cropping systems have undergone intensive modification over the last century through the development of high-yielding cultivars, the use of fertilizers, pesticides, and herbicides, and the adoption of advanced machinery. However, these high-input, annually disturbed systems negatively affect soil and the environment, reducing carbon sequestration, increasing nutrient leaching, and contributing to greater greenhouse gas emissions than perennial cropping systems [1–4].

Perennial crops possess morphological and physiological traits that make them a sustainable alternative to annuals. Perennial grasses can regrow from underground stems (rhizomes) or from axillary buds at the base of shoots, enabling rapid regrowth after winter or other stress events such as drought, and prolonged growth throughout the season. Their extensive root systems, combined with year-round soil coverage and minimal soil disturbance, reduce erosion and nutrient leaching [5]. Moreover, continuous root growth and biomass turnover enhance long-term soil carbon storage and nutrient cycling, benefiting overall soil health [6–9]. Despite their promise, the limited availability of high-yielding perennial grain crops with broad climate adaptation and favorable agronomic traits remains a key constraint for transitioning to sustainable perennial agriculture.

A perennial grain crop must maintain vegetative and reproductive growth over multiple growing seasons at the local farm site. In cold-temperate climates, the crop must recognize and adapt to seasonal changes and frost [10]. Thus, it must respond to environmental signals, such as temperature and day length, which regulate regrowth, flowering, and the induction of dormancy and frost tolerance, and must reach seed maturity before the end of the growing season. Avoiding frost damage is critical for plant survival. Plants employ several physiological and cellular mechanisms to enhance frost tolerance, including osmotic adjustment and membrane stabilization [11]. However, unstable winter conditions characterized by freeze–thaw cycles can reduce frost tolerance, cause tissue damage, and increase susceptibility to biotic stresses. As a result, perennial plants are exposed to multiple interacting stresses and require a range of adaptive traits to survive. Such physiological and developmental requirements make breeding perennial grains particularly challenging, as traits for perennial growth, stress tolerance, and timely reproduction must be combined with agronomically desirable traits such as high yield and synchronized development.

Progress in developing perennial cereal crops is achieved through two main breeding strategies. The first strategy, domestication, involves selecting plants with desirable traits from a wild perennial species and improving them through open pollination or repeated crossing over multiple generations. This approach is applied in the breeding of the perennial wheat relative *Thinopyrum intermedium* (intermediate wheatgrass, Kernza®) and is increasingly combined with genomic selection to enhance traits such as seed weight and resistance to shattering [12–17]. The second strategy, wide hybridization, has been successfully used in the development of perennial rice [18,19] and is also applied in perennial sorghum [20,21] for subtropical and mild temperate climates. In this approach, traits associated with perennial growth are transferred into an annual cereal cultivar by crossing it with a perennial wild relative to generate stable introgressions of chromosomal segments carrying important genes. The breeding of perennial cereals adapted to cold-temperate climates is still at an early stage, and the perennial barley relative *Hordeum bulbosum* has recently been suggested as a promising candidate [22]. *H. bulbosum* is the closest perennial relative of cultivated barley [23], which is one of the most important cereals in northern agricultural regions. *Hordeum bulbosum* occurs in both diploid and tetraploid forms [21]. The diploid form has the same chromosome number as cultivated barley (2n = 2x = 14), whereas the tetraploid form has 2n = 4x = 28. Despite their different ploidy levels, the two forms are not morphologically distinct and are not recognized as separate taxa. *H. bulbosum* has a large genome of approximately 4 Gb (1C), representing about 80% of the genome size of barley [24].

Due to the close genetic relationship with barley, *H. bulbosum* has been crossed with barley and used in breeding, primarily to improve pathogen resistance [25–29] and has also been valuable for mapping genes controlling resistance to biotic stresses [30,31]. In these hybrids, meiotic pairing and recombination occur, although recombination is greatly reduced compared to intraspecific recombination within barley [30]. By crossing tetraploid

and diploid forms of *H. bulbosum* with diploid barley, followed by further hybrid crossings and chromosome doubling, segments of *H. bulbosum* chromosomes have been introgressed into the barley parental background [29,32–35]. This extensive work was carried out by several researchers, with most studies published between the 1980s and the 2000s. There were many challenges to achieving successful crosses and introgressions, including low pollen fertility, elimination of *H. bulbosum* chromosomes, and low recombination rates between the genomes of the two species. The resulting barley–*H. bulbosum* introgression lines (ILs) were derived from crosses between four spring barley cultivars—Emir, Golden Promise, Vada (European origin), and Morex (North American origin)—and twelve *H. bulbosum* clones. The ILs were donated to the gene bank Nordic Genetic Resource Center (NordGen). To our knowledge, no winter barley cultivars have been used as parents for creating similar introgression lines.

As a species, *H. bulbosum* is distributed across a broad geographic range extending from the Mediterranean region of southern Europe to temperate regions of Central Asia, and from elevations of 10 to 3000 m [36], and is thus exposed to a range of climates. Some of the *H. bulbosum* clones used to develop the ILs originate from southern Europe, and others have unknown origins. These interspecific crosses generated a diverse set of ILs containing introgressed *H. bulbosum* segments representing parts of all barley chromosomes. Many ILs carry single terminal segments, while others have two or three terminal segments, either on different chromosomes or on both arms of the same chromosome [37]. The introgressions have been characterized using genetic markers [29], genomic in situ hybridization [28,38], and genotyping-by-sequencing (GBS) to investigate segment positions and genetic diversity [37].

As the annual barley parents, the ILs are self-fertilizing and highly homozygous. They maintain a stable chromosome number over generations and are multiplied by selfing [29,34]. These ILs have not yet been evaluated for perennial growth habit. The ILs possess several attributes that make them highly interesting for evaluation as potential genetic resources in the development of perennial barley adapted to cold-temperate climates. First, our previous study of *H. bulbosum* accessions [22] demonstrated high winter survival and strong regrowth over 4 years under field conditions characterized by seasonal variation, frost in fall, winter, and spring, and days in summer. Second, the main part of the genome of each IL is inherited from barley and thus retains many of the agronomic traits acquired through barley domestication and breeding, which may be advantageous for the development of perennial barley. Third, *H. bulbosum* and barley exhibit a high level of genome synteny [37,39–41]. By comparing the introgressed *H. bulbosum* segments with the well-characterized barley genome [42], gene functions can be inferred for the introgressed segments. Collectively, these attributes provided a strong rationale for investigating the effects of introgressed *H. bulbosum* chromosomal segments on perenniality. The objective of this study was to investigate the potential of 151 barley–*H. bulbosum* ILs as genetic resources for developing perennial barley. We evaluated these ILs for traits associated with perennial growth habit, including vegetative and reproductive growth, under field conditions in central Sweden (cold-temperate climate) in separate spring- and fall-planted field experiments conducted in different years. The same ILs were also evaluated over two simulated annual cycles in a climate chamber to compare their performance under controlled and milder growing conditions. The use of a well-characterized set of ILs enabled us to associate phenotypic variation with the distinct introgressed *H. bulbosum* segments. We hypothesized that some ILs would show phenotypes associated with perennial growth and that the observed phenotypic variation among ILs would be associated with the chromosomal origin of the introgressed segments.

2. Materials and Methods

2.1. Plant Material

In the present study, seeds of 151 barley–*H. bulbosum* ILs and the parental cultivars Emir, Golden Promise, and Morex were obtained from the Nordic Genetic Resource Center (NordGen), Alnarp, Sweden (Table S1). However, we did not have access to the *H. bulbosum* parental lines. The most common barley parents of the ILs were the spring cultivars Emir, Golden Promise, and Morex. The most frequent *H. bulbosum* donors were A17/1, HB2032, and 2920/4 [43,44].

Most ILs were reported to carry introgressions on single chromosomes, either on the short or long arm (Table S1) [29,37]. Some ILs carried two or three introgressed segments located on the same or on different chromosomes. The *H. bulbosum* genome coverage of the introgressed segments ranged from 6% to 24%, based on the base–pair sequence for each chromosome [37]. All types of chromosome introgressions were represented in the set of ILs used in the present study.

2.2. Evaluation Under Field Conditions

2.2.1. Field Trial 1—Spring Planting

Seeds of 151 introgression lines (ILs) and the parental cultivars were germinated in 1.5 L pots containing a low-nutrient commercial potting soil (S-jord; Hasselfors Garden, Örebro, Sweden). For each IL, three seeds were sown per pot in three pots. Plants were grown in a greenhouse at the Plant Cultivation Facility, Uppsala BioCenter, SLU, Uppsala, Sweden, under a 16 h light/8 h dark photoperiod, with supplementary metal halide lighting and a day/night temperature regime of 22/20 °C. Three weeks after sowing, plants of equal size for each IL were selected by removing the other two plants in each pot. Eight-week-old plants were then subjected to a vernalization treatment for 6 weeks at +4 °C under an 8 h photoperiod at 100 µmol m^{-2} s^{-1}. Before planting them in the field, plants were acclimatized in the greenhouse for two weeks.

In early May 2013, three plants of equal size of each IL and parental cultivar were selected and planted in clay soil in a farmer's field located north of Uppsala, central Sweden (60°00′ N, 17°42′ E, Figure 1). The experiment was arranged in a randomized complete block design with three blocks, each containing one plant per IL and parental cultivar. Plant spacing was 0.5 m within rows and 0.6 m between rows. The field site was managed organically. Weeds were removed manually, no pesticides or herbicides were applied, and a low level of animal manure was used as fertilizer. Climatic conditions at the cultivation site from 2013 to 2016 are shown in Figure S1.

In September, 14–16 weeks after planting, when the spikes had reached full maturity, the plants were cut off 10 cm from the base and evaluated for tiller number, shoot dry weight, and seed number per plant (Table 1). Four weeks after the harvest, regrowth was evaluated based on the number of new tillers. The ILs were also observed for winter survival in spring 2014.

Table 1. Traits and calculated variables obtained from the evaluation of barley–*H. bulbosum* introgression lines in the field in Uppsala, Sweden (60°00′ N, 17°42′ E), and in the climate chamber.

Trait	Abbreviation	Activity	Season
In field			*Natural season*
Number of tillers per plant at harvest	TNH-f	Harvest	Late summer
Number of seeds per plant	SN-f	Harvest	Late summer
Shoot dry weight per plant (g)	SDW-f	Harvest	Late summer
Number of tillers per plant at regrowth	TNR-f	Evaluation	Fall
Relative regrowth ((TNR-f − TNH-f)/TNH-f))	ΔTNR-f	Calculated	
Plants with spikes	PSP-f	Evaluation	Fall

Table 1. *Cont.*

Trait	Abbreviation	Activity	Season
In climate chamber			*Simulated season*
			1st growing season
Number of tillers per plant at harvest	TNH-c	Harvest	Fall
Number of tillers per plant at regrowth	TNR1-c	Evaluation 1, E1	Late fall
Relative regrowth ((TNR1-c − TNH-c)/TNH-c))	ΔTNR1-c	Calculated	
			2nd growing season
Number of tillers with flag leaves per plant	FN-c	Evaluation 2, E2	Spring
Number of tillers with spikes per plant	SPN-c	Evaluation 2, E3	Spring
Number of tillers per plant in 2nd summer	TNR2-c	Evaluation 3, E3	Early summer
Relative continued regrowth ((TNR2-c − TNH-c)/TNH-c))	ΔTNR2-c	Calculated	
Spikes on plant (presence/absence)	SPP-c	Evaluation 4, E4	Late summer

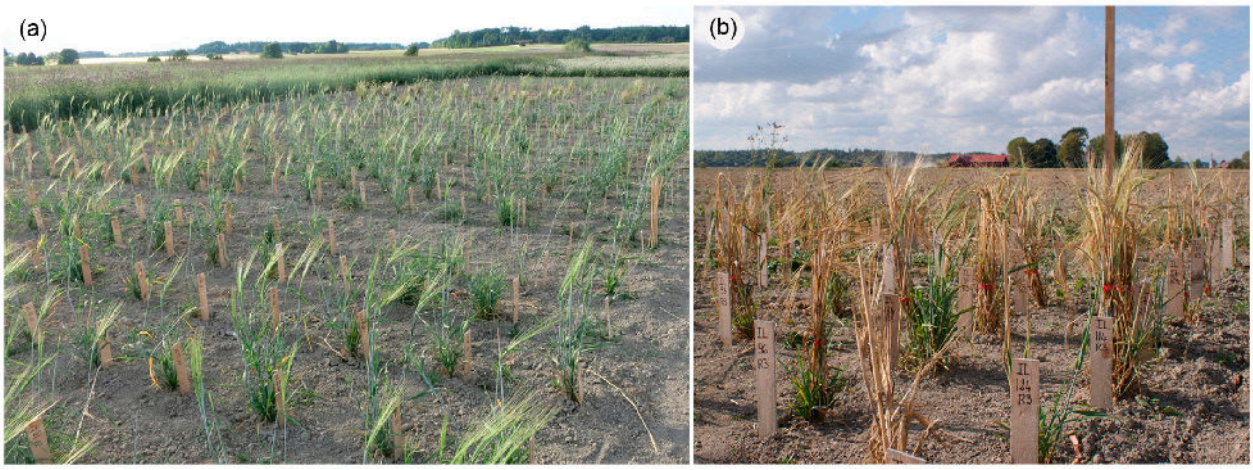

Figure 1. Field trial 1 in Uppsala, Sweden (60°00′ N, 17°42′ E) showing barley-*Hordeum bulbosum* introgression lines (**a**) in summer and (**b**) in fall. Photographs taken by Anna Westerbergh.

2.2.2. Field Trial 2—Late Summer Planting

To further evaluate the ILs under field conditions, an additional field trial was established in 2015 using the same ILs and experimental design as in Field Trial 1. In this trial, non-vernalized, two-month-old plants were transplanted in mid-August and evaluated in early October. During this growth period, under conditions with gradually shorter days, tiller and spike development were recorded to evaluate the plants' ability to flower. Winter survival was evaluated in spring 2016.

2.3. Evaluation Under Simulated Seasonal Changes in Controlled Climate Conditions

To simultaneously evaluate the large set of ILs for their responses to seasonal changes in climate and their ability to regrow over two simulated consecutive year cycles, single plants of the ILs and two plants of each parental cultivar (Morex, Emir, Golden Promise, and Vada) were cultivated together in a single climate chamber according to a randomized experimental design. The ILs could therefore be evaluated under the same climate chamber conditions while using pots of a reasonable size. The chamber (4.6 m^2, 2.4 m high; model BDW50, Conviron, Winnipeg, MB, Canada) was located at the Phytotron Plant Cultivation Facility, Uppsala BioCenter, SLU Uppsala, Sweden. Seasonal changes were simulated by varying temperature, photoperiod, and light intensity in a climate chamber (Figure 2).

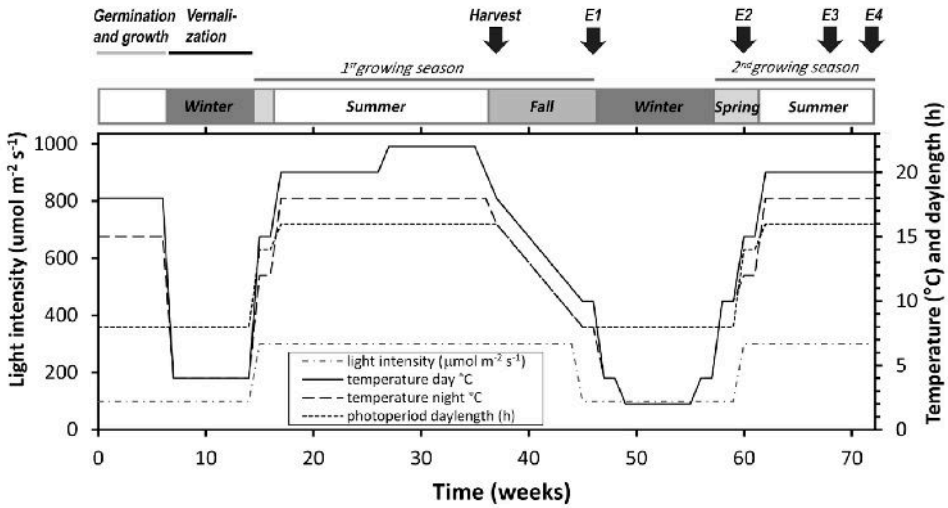

Figure 2. Treatment protocol for cultivation of barley—*H. bulbosum* ILs in a climate chamber to evaluate plant regrowth responses under simulated seasonal changes during two year-cycles. Tillers were cut at harvest, and plant traits were evaluated at four time-points (E1–E4).

Seeds of the ILs and the parental cultivars were germinated and cultivated in 3 L pots (16 cm in diameter, 19 cm in height) with a low-nutrient commercial soil potting mix (S-jord, Hasselfors Garden, Örebro, Sweden). A complete nutrient solution (Wallco nutrient solution, Cederroth International AB, Arlöv, Sweden) containing 0.65 mM P, 6 µM Fe, 7.3 mM N as NH_4NO_3, 2.2 mM K, 0.25 mM S, 0.15 mM Ca, 0.33 mM Mg, 7.3 µM Mn, 18 µM B, 0.92 µM Zn, 0.47 µM Cu, and 0.008 µM Mo was supplied during the growth phase, while deionized water was provided during the cold period.

After six weeks, the plants were vernalized as described above. Following a 21-week growth period under long-day conditions, the temperature, day length, and light intensity were gradually reduced (Figure 2). At harvest, the tillers were cut approximately 20 cm from the base, and regrowth was assessed after nine weeks at time-point E1. The plants were then exposed to cold conditions, followed by simulated spring and summer. Plant growth traits were evaluated during the second growing season at three time points (E2–E4) as described in Table 1. In addition, the morphology of the shoot base and the tiller growth was observed throughout the experiment.

2.4. Data Analysis

Normality of residuals and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. When the assumptions were met, differences among groups were analyzed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. When homogeneity of variances was not met, Welch's one-way ANOVA followed by the Games–Howell post hoc test was used. Analysis of Means (ANOM) was used to identify group means that differed significantly from the overall mean using Minitab Statistical Software (version 22; Minitab, LLC, State College, PA, USA). Statistical significance was set at $p < 0.05$. To analyze and visualize relations among ILs based on all studied traits, principal component analysis (PCA) was conducted using the software SIMCA 14.1 (Umetrics AB, Umeå, Sweden). Pearson's correlation coefficients were used to assess linear associations among traits. The broad-sense heritability H^2 was estimated for traits studied in the field. Pearson's correlation coefficients and H^2 were analyzed using R (version 4.5.1; 2025-06-13 ucrt), packages "inti" and "variability", in RStudio (version 2025.9.1.401; Posit Software, PBC, Boston, MA, USA).

$$H^2 = \frac{\delta_g^2}{\delta_p^2} \quad (1)$$

where δ_g^2 is the genotypic variance, δ_p^2 is the phenotypic variance and $\delta_p^2 = \delta_g^2 + \frac{\delta_e^2}{r}$ in which r is the number of replicates.

3. Results

3.1. Growth Before Harvest

3.1.1. Field Trial 1—Spring Planting

The ILs showed a large variation in tiller number per plant (TNH-f) at harvest in Field Trial 1, 2013. One reason for this variability is that each IL represents a unique genotype, carrying a distinct *H. bulbosum* introgression despite sharing most of the genetic background of the same barley parent cultivar.

Among the ILs grouped by different barley cultivar parental backgrounds, the ILs derived from Emir, Golden Promise, and Morex had, on average, 13.6, 11.7, and 4.2 TNH-f, respectively (Figure 3). ILs with Morex as the parent were significantly different from the ILs with Emir or Golden Promise as the barley parent. The average TNH-f for ILs derived from each parental cultivar was higher than that of the corresponding parental cultivar. The parental cultivar Vada did not survive until harvest in the fall. Based on the most common *H. bulbosum* parents 2920/4, A17/1, and HB2032 of the ILs, TNH-f was on average 12.0, 13.6, and 9.2, respectively, and the ILs with the HB2032 parent differed significantly from the A17/1 parent (Figure 4). A similar pattern was observed for seed number per plant (SN-f) and shoot dry weight (SDW-f) as for TNH-f, both between ILs with different barley parents and between ILs with different *H. bulbosum* parents (Figures 3 and 4). No significant difference was observed in regrowth (TNR-f) among ILs with different barley parents or among ILs with different *H. bulbosum* parents.

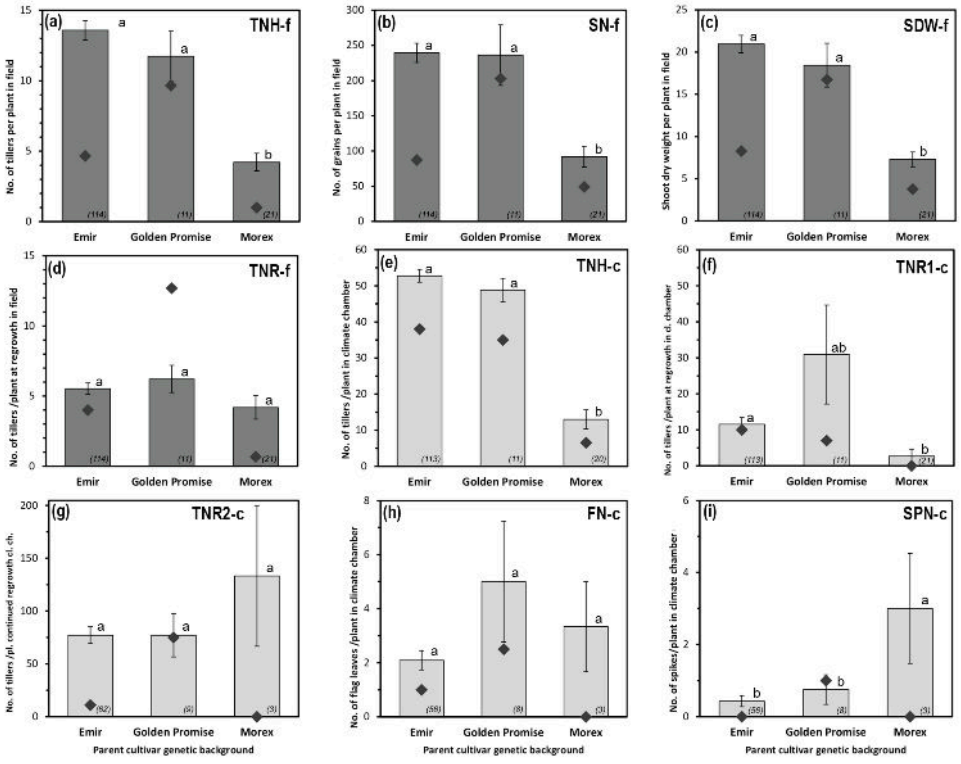

Figure 3. Growth, regrowth, and seed traits of ILs grouped according to their parental barley cultivar genetic backgrounds Emir, Golden Promise, and Morex evaluated in field and climate chamber. Field

traits: (**a**) number of tillers per plant (TNH-f), (**b**) number of seeds per plant (SN-f), and (**c**) shoot dry weight per plant (SDW-f) at harvest, and (**d**) number of tillers after regrowth (TNR-f). Climate chamber traits: (**e**) number of tillers per plant at first-season harvest (TNH-c), (**f**) number of tillers at regrowth at time point E1 (TNR1-c), (**g**) number of tillers at time point E3 (TNR2-c), (**h**) number of tillers with flag leaves per plant at E2 (FN-c), and (**i**) number of tillers with spikes per plant at E3 (SPN-c). Values are means ± SE. Mean values that do not share the same letter are significantly different among groups according to one-way ANOVA followed by Tukey's HSD test (panels (**d**,**g**,**i**)) or Welch's one-way ANOVA followed by the Games–Howell post hoc test (panels (**a**–**c**,**e**,**f**,**h**)) ($p < 0.05$). Numbers in parentheses within the bars represent the number of ILs studied, and diamonds represent the mean of the corresponding barley cultivar parent. For detailed information on traits and time points, see Table 1.

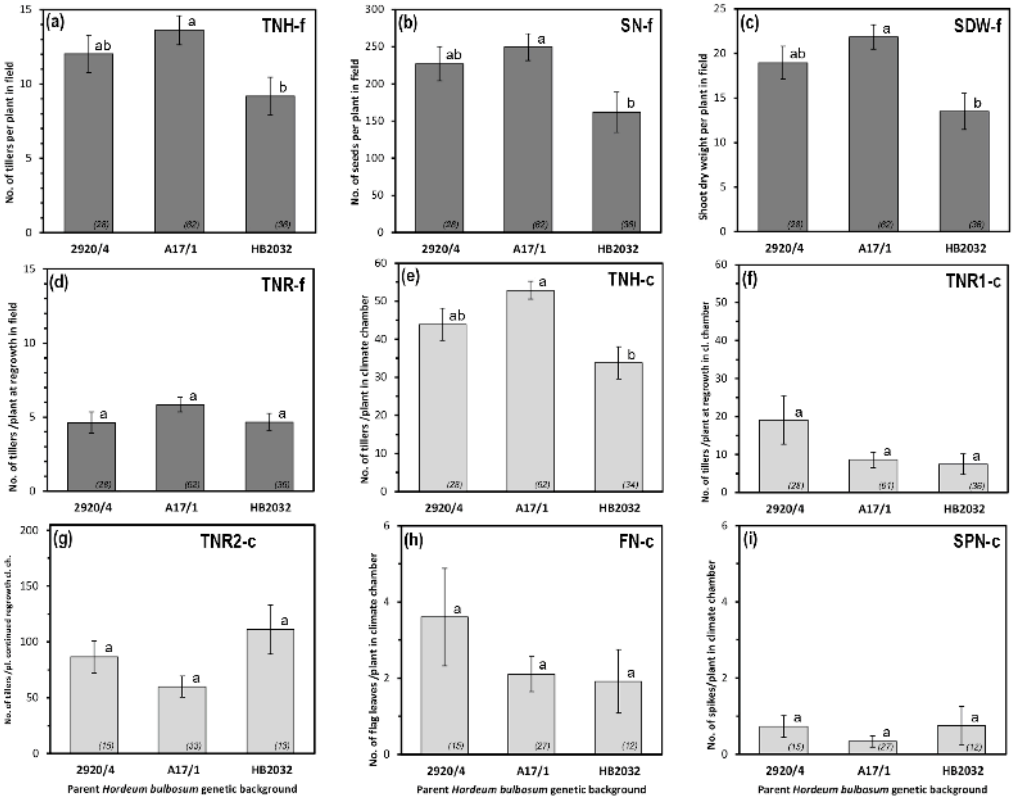

Figure 4. Growth, regrowth, and seed traits studied in ILs grouped according to their *H. bulbosum* parent genetic backgrounds 2920/4, A17/1, and HB2032 evaluated in field and climate chamber. Field traits: (**a**) number of tillers per plant (TNH-f), (**b**) number of seeds per plant (SN-f), (**c**) shoot dry weight per plant (SDW-f) at harvest, and (**d**) number of tillers after regrowth (TNR-f). Climate chamber traits: (**e**) number of tillers per plant at first-season harvest (TNH-c), (**f**) number of tillers at regrowth at time point E1 (TNR1-c), (**g**) number of tillers at time point E3 (TNR2-c), (**h**) number of tillers with flag leaves per plant at E2 (FN-c), and (**i**) number of tillers with spikes per plant at E3 (SPN-c). Values are means ± SE. Mean values that do not share the same letter are significantly different among groups according to one-way ANOVA followed by Tukey's HSD test (panel (**e**)) or Welch's one-way ANOVA followed by the Games–Howell post hoc test (panels (**a**–**d**,**f**–**i**)) ($p < 0.05$). Numbers in parentheses within the bars represent the number of ILs studied, and diamonds represent the mean of the corresponding barley cultivar parent. For detailed information on traits and time points, see Table 1.

3.1.2. Controlled Climate Conditions

Tiller number per plant (TNH-c) varied among the ILs also under controlled climate conditions and was higher than in the field. The ILs derived from Morex (13.0 tillers per plant) produced a significantly lower number of tillers before harvest (TNH-c) than ILs derived from Emir (52.7 tillers per plant) and from Golden Promise (48.8 tillers per plant)

(Figure 3). Similarly, in the climate chamber, the average TNH-c of the ILs with different barley parents was higher than that of their corresponding barley parent (Figure 3). ILs with the *H. bulbosum* parent HB2032 had the lowest TNH-c in the climate chamber (Figure 4).

3.2. Regrowth After Harvest

3.2.1. Field Trial 1—Spring Planting

No significant difference was observed in regrowth in the field (TNR-f) among ILs with different barley parents or among ILs with different *H. bulbosum* parents (Figures 3 and 4). Notably, some ILs showed strong relative regrowth after harvest in the field (ΔTNR-f, Figure 5). These ILs had an Emir or Golden Promise parental genetic background. Similar to the annual parent cultivars, many other ILs showed weak regrowth, and some ILs were unable to regrow any new tillers after harvest, which was reflected as a −1 value for ΔTNR-f in Figure 5. Like the parental cultivars, none of the ILs survived the winter.

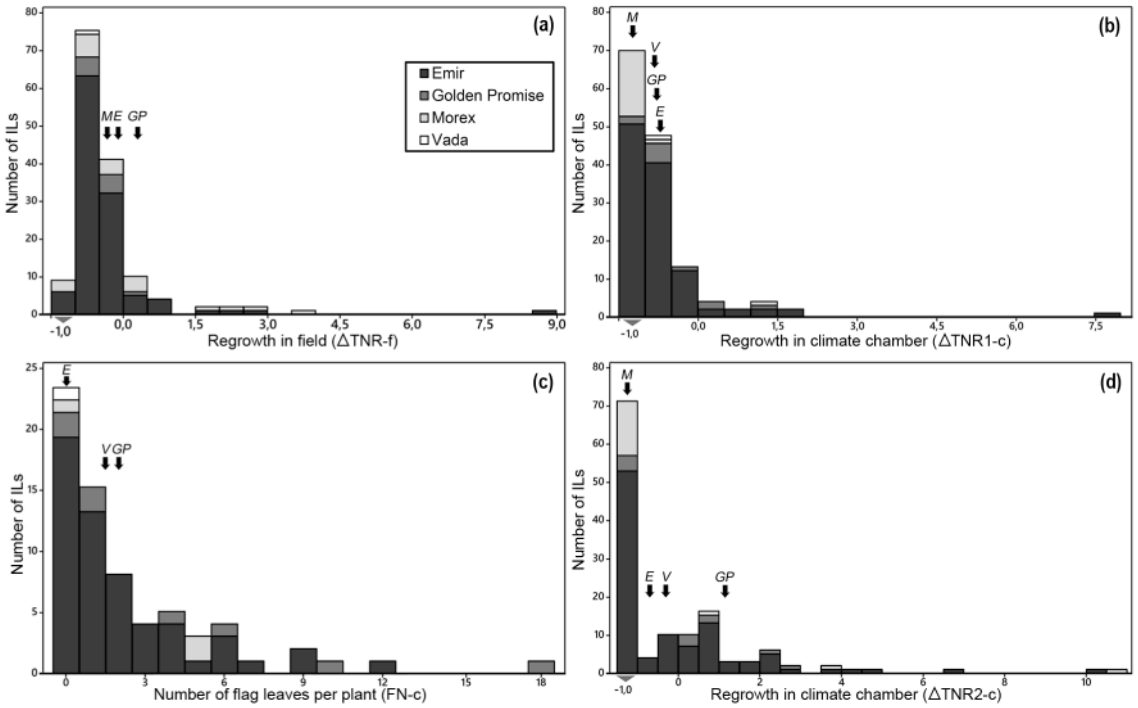

Figure 5. Distribution of barley—*H. bulbosum* ILs with different barley parental cultivar genetic backgrounds, and means of parental cultivars Emir (E), Morex (M), Golden Promise (GP), and Vada (V) for regrowth traits in field and climate chamber, indicated by arrows. (**a**) relative regrowth in the field (ΔTNR-f), (**b**) relative regrowth in the climate chamber at E1 during simulated late fall (ΔTNR1-c), (**c**) number of tillers with flag leaves at E2 after the simulated winter (FN-c), and (**d**) continued relative regrowth in the climate chamber at E3 late in the second growing season (ΔTNR2-c). No regrowth is indicated by a value of −1, while regrowth with a tiller number equal to the tiller number before harvest is indicated by a value of 0. For detailed information on regrowth traits, see Table 1.

3.2.2. Controlled Climate Conditions

Regrowth in the climate chamber was generally low, especially for the ILs with Emir as the barley parent and A17/1 as the *H. bulbosum* parent (Figures 3 and 4). About 46% of the ILs did not show relative regrowth (ΔTNR1-c) in the climate chamber at E1 after the harvest in the first growing season and subsequently died, as indicated by the high number of ILs with a value of −1 for ΔTNR1-c in Figure 5. Most of the ILs with Morex as the parental cultivar belonged to that group. The remaining ILs showed a distribution pattern in regrowth at E1 similar to that found in the field, with some producing a higher number

of tillers than during the first growing season before harvest (ΔTNR1-c > 0). Among the ILs that showed regrowth, those with Emir as the parental cultivar were the most abundant.

Almost all ILs that showed regrowth at E1 survived the simulated winter. At E2 in the second growing season, 67% of these ILs had produced reproductive tillers with flag leaves (FN-c, Figure 5). No significant differences were observed in FN-c or SPN-c (number of spikes per plant) among ILs with different *H. bulbosum* parents (Figure 4), as well as for FN-c among ILs with different barley parents (Figure 3). Morex ILs, however, showed, a higher SPN-c than ILs with Emir or Golden Promise as barley parents. The ILs continued to show medium to strong regrowth of tillers at E3 late in the second simulated growing season (ΔTNR2-c) and produced higher numbers of tillers (TNR2-c) than during the first growing season before harvest (TNH-c; Figure 4).

The Morex parental cultivar did not survive the first simulated season in the climate chamber, while the Emir, Golden Promise, and Vada parents survived until the end of the second simulated growing season, with Golden Promise showing the highest ΔTNR2-c (Figure 5). About 18% of the ILs showed higher ΔTNR2-c than Golden Promise. Compared to many ILs, Emir, Golden Promise, and Vada showed a relatively low transition from the vegetative to reproductive stage, measured by the number of tillers with flag leaves per plant (FN-c).

3.3. Correlations Between Traits Studied in the Field and in the Climate Chamber

Pairwise comparisons between growth and regrowth traits studied in the field and in the climate chamber were analyzed using Pearson's correlation coefficients (Table 2). In the field, strong positive correlation was observed between all growth traits (SDW-f, SN-f, TNH-f (r = 0.89–0.94), and between the regrowth traits TNR-f and ΔTNR-f (r = 0.62), while the tiller number before harvest (TNH-f) and after harvest (TNR-f) showed no correlation (r = 0.08). In the climate chamber, all regrowth traits related to tiller production (TNR1-c, ΔTNR1-c, TNR2-c, ΔTNR2-c) were strongly positively correlated (r = 0.60–0.78). The reproductive regrowth trait FN-c showed moderate correlations with the tiller regrowth traits (r = 0.30–0.55), while SPN-c showed weak to moderate correlations with these traits (r = 0.18–0.35). When corresponding traits were compared between the field and the climate chamber, the number of tillers produced before harvest (TNH-f and TNH-c) showed a moderate positive correlation (r = 0.49), indicating that the relative growth performance of the ILs was broadly consistent during the initial growth across the two environments. Tiller number after the first harvest (TNR-f and TNR1-c) showed no correlation (r = 0.09).

Table 2. Correlation analysis between growth traits studied in the field; number of tillers per plant at harvest (TNH-f), number of seeds per plant (SN-f), shoot dry weight per plant (SDW-f), number of tillers per plant at regrowth (TNR-f) and relative regrowth (ΔTNR-f), and between traits studied in the climate chamber number of tillers per plant at first-season harvest (TNH-c), number of tillers at regrowth at time point E1 (TNR1-c), relative regrowth at time point E1 (ΔTNR1-c), number of tillers at time point E3 (TNR2-c), relative regrowth at time point E3 (ΔTNR2-c), number of tillers with flag leaves per plant at E2 (FN-c), and number of tillers with spikes per plant (SPN-c). Values show Pearson's correlation coefficients (r). Significant correlations are denoted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Field			**Climate Chamber**			**Field—Climate Chamber**		
Trait 1	**Trait 2**	***r***	**Trait 1**	**Trait 2**	***r***	**Trait 1**	**Trait 2**	***r***
TNH-f	TNR-f	0.084	TNH-c	TNR1-c	−0.019	TNH-f	TNH-c	0.491 ***
SN-f	TNH-f	0.921 ***	TNH-c	TNR2-c	0.088	TNR-f	TNR1-c	0.087
SN-f	TNR-f	0.066	TNR1-c	TNR2-c	0.726 ***	TNR-f	TNR2-c	0.206 *
SDW-f	TNH-f	0.886 ***	ΔTNR1-c	TNH-c	−0.22 **	ΔTNR-f	ΔTNR1-c	−0.014

Table 2. *Cont.*

Field			Climate Chamber			Field—Climate Chamber		
Trait 1	**Trait 2**	***r***	**Trait 1**	**Trait 2**	***r***	**Trait 1**	**Trait 2**	***r***
SDW-f	SN-f	0.940 ***	ΔTNR1-c	TNR1-c	0.624 ***	ΔTNR-f	ΔTNR2-c	0.128
SDW-f	TNR-f	0.126	ΔTNR1-c	TNR2-c	0.399 ***			
ΔTNR-f	TNH-f	−0.261 **	ΔTNR1-c	ΔTNR2-c	0.784 ***			
ΔTNR-f	SN-f	−0.231 **	ΔTNR1-c	FN-c	0.303 ***			
ΔTNR-f	SDW-f	−0.215 **	ΔTNR1-c	SPN-c	0.176 *			
ΔTNR-f	TNR-f	0.622 ***	ΔTNR2-c	TNH-c	−0.155			
			ΔTNR2-c	TNR1-c	0.596 ***			
			ΔTNR2-c	TNR2-c	0.745 ***			
			ΔTNR2-c	FN-c	0.382 ***			
			ΔTNR2-c	SPN-c	0.222 **			
			FN-c	TNH-c	0			
			FN-c	TNR1-c	0.551 ***			
			FN-c	TNR2-c	0.476 ***			
			FN-c	SPN-c	0.651 ***			
			SPN-c	TNH-c	0.004			
			SPN-c	TNR1-c	0.352 ***			
			SPN-c	TNR2-c	0.297 ***			

3.4. Heritability

In the field, the broad-sense heritability (H^2) for the ILs and parents combined was high for all growth traits studied at harvest (0.55–0.64), with TNH-f showing the highest value (Table 3). The regrowth traits TNR-f and ΔTRN-f showed moderate H^2 values (0.38– 0.45).

Table 3. Genetic variance (δ_g^2), environmental variance (δ_e^2), and broad-sense heritability (H^2), of growth traits studied in the field: number of tillers per plant at harvest (TNH-f), number of seeds per plant (SN-f), shoot dry weight per plant (SDW-f), number of tillers per plant at regrowth (TNR-f), and relative regrowth (ΔTNR-f).

Statistics	**Traits**				
	TNH-f	**SN-f**	**SDW-f**	**TNR-f**	**ΔTNR-f**
Genetic variance, δ_g^2	34.0	10,876.4	62.3	8.2	0.72
Environmental variance, δ_e^2	50.9	23,019.6	135.2	35.7	2.30
Heritability, H^2	0.64	0.56	0.55	0.38	0.45

3.5. Growth and Regrowth Traits Affected Distribution Patterns of ILs in PCA

To assess the diversity among the ILs and identify ILs with phenotypes important for the development of perennial barley, we performed multivariate analysis of a dataset based on growth and regrowth traits evaluated in Field Trial 1 planted in spring 2013 (Figure 6). We also combined this dataset with the results from the climate chamber experiment (Figure 7). The PCA biplots highlighted the relative importance of the measured and calculated plant traits in the field and the climate chamber.

In the PCA based on the field data, the first principal component explained 60% of the total variation. It was strongly associated with TNH-f, SDW-f, and the reproductive trait SN-f (Figure 6). Meanwhile, the regrowth trait TNR-f and the calculated regrowth trait ΔTNR-f were associated with the second principal component, which explained 29% of the variation (Figure 6).

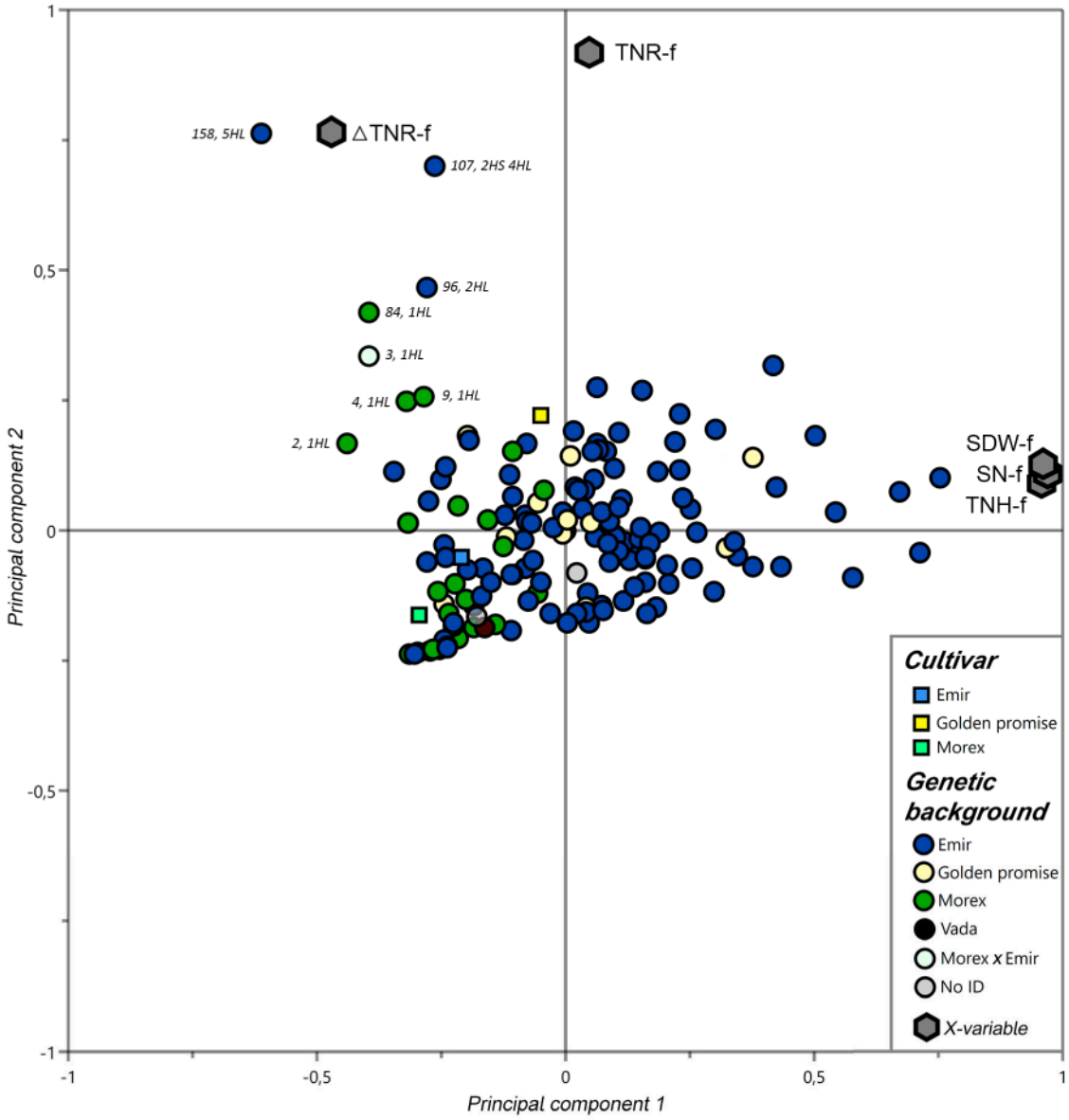

Figure 6. Principal Component Analysis illustrating the distribution of ILs with different barley genetic backgrounds and their parental cultivars, together with the loadings of the studied traits evaluated in Field Trial 1 planted in spring 2013; number of tillers per plant at harvest (TNH-f), number of seeds per plant (SN-f), shoot dry weight per plant (SDW-f), number of tillers per plant at regrowth (TNR-f), relative regrowth (ΔTNR-f). The first component explained 60% of the variation, and the second component explained 29% of the variation. Eight of the ILs are labeled by their accession SLU ID number, and their chromosomal introgression types are indicated by the barley chromosome number and chromosome arm, short (S) or long (L) (Table S1).

In the PCA based on the combined dataset from the field and the climate chamber trials, the first principal component explained 37% of the total variation. It was positively associated with the growth traits studied at harvest, both in the field (TNH-f, SN-f, SDW-f) and in the climate chamber (TNH-c). In contrast, it was negatively associated with regrowth traits such as ΔTNR-f and FN-c (Figure 7). The second principal component, which explained 20% of the variation, was associated with several regrowth traits (TNR1-c, TNR2-c, ΔTNR1-c, and ΔTNR2-c). Most ILs formed a single major cluster, particularly along PC1, while several ILs were separated from this cluster and exhibited divergent phenotypes, indicating substantial diversity in the studied growth and regrowth traits.

(Figures 6 and 7). Some divergent phenotypes were positively associated with the regrowth traits, while others were more closely associated with growth traits.

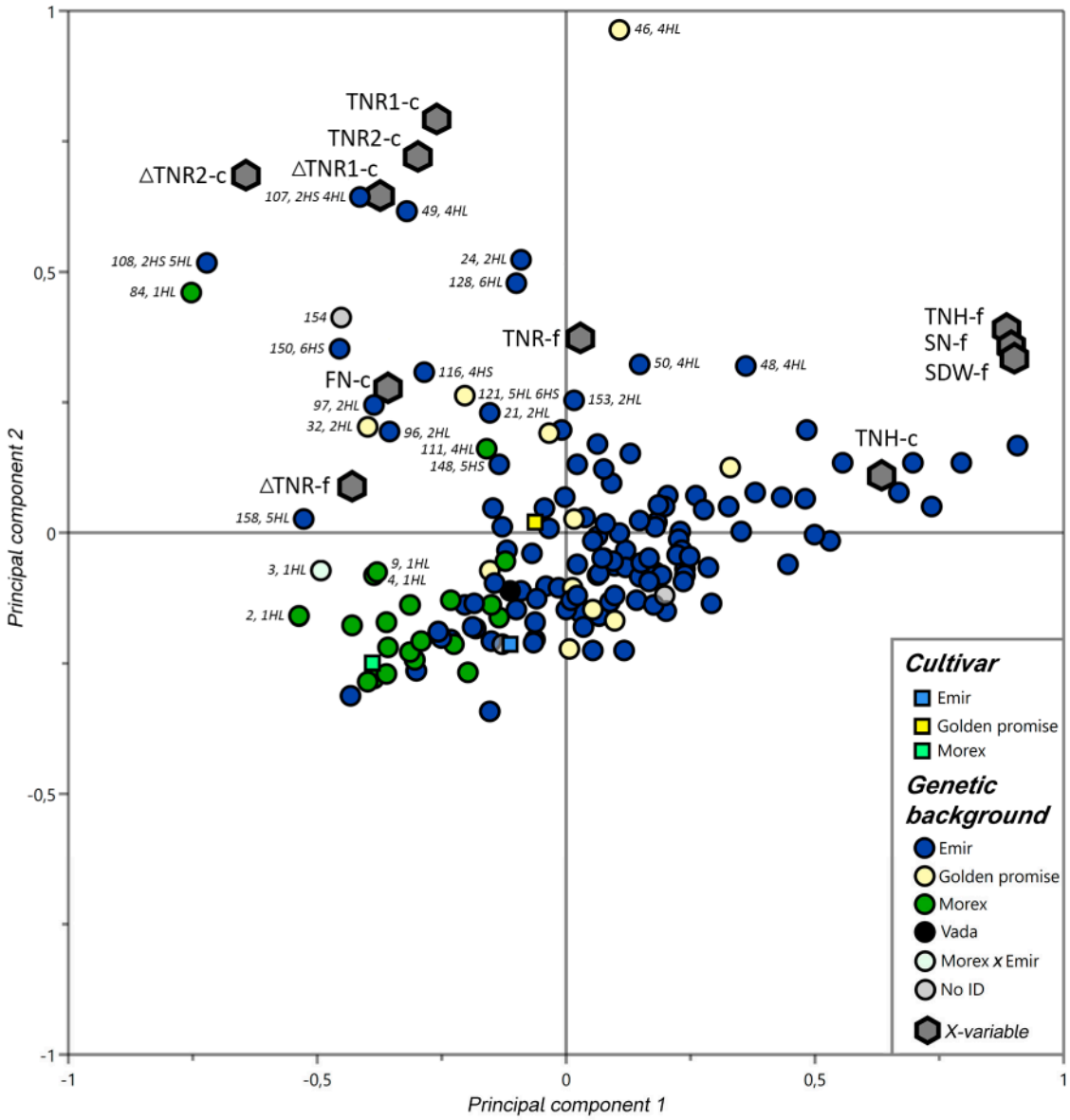

Figure 7. Principal Component Analysis illustrating the distribution of ILs with different barley genetic backgrounds and their parental cultivars, together with the loadings of the studied traits evaluated in Field Trial 1 planted in spring 2013 and in the climate chamber. Field traits: number of tillers per plant (TNH-f), number of seeds per plant (SN-f), and shoot dry weight per plant (SDW-f) at harvest, number of tillers after regrowth (TNR-f), and relative regrowth (ΔTNR-f). Climate chamber traits: number of tillers per plant at first-season harvest (TNH-c), number of tillers at regrowth at time point E1 (TNR1-c), relative regrowth at time point E1 (ΔTNR1-c), number of tillers at time point E3 (TNR2-c), relative regrowth at time point E3 (ΔTNR2-c), number of tillers with flag leaves per plant at E2 (FN-c), and number of tillers with spikes per plant at E3 (SPN-c). The first component explains 37% of the variation, and the second component explains 20%. Twenty-five of the ILs are labeled by their accession ID-SLU number, and their chromosomal introgression types are indicated by the barley chromosome number and chromosome arm, short (S) or long (L) (Table S1).

FN-c at the beginning of the second growing season, studied as a reproductive trait following regrowth in the climate chamber experiment, was associated with the regrowth traits (Figure 7). This indicates that many of the ILs exhibiting regrowth also produced

reproductive tillers. In fact, the majority of the ILs that were distributed toward the regrowth trait variables in the PCA had a high FN-c at E2. This group of ILs also produced spikes at E4, at the end of the second simulated growing season (Figure 8).

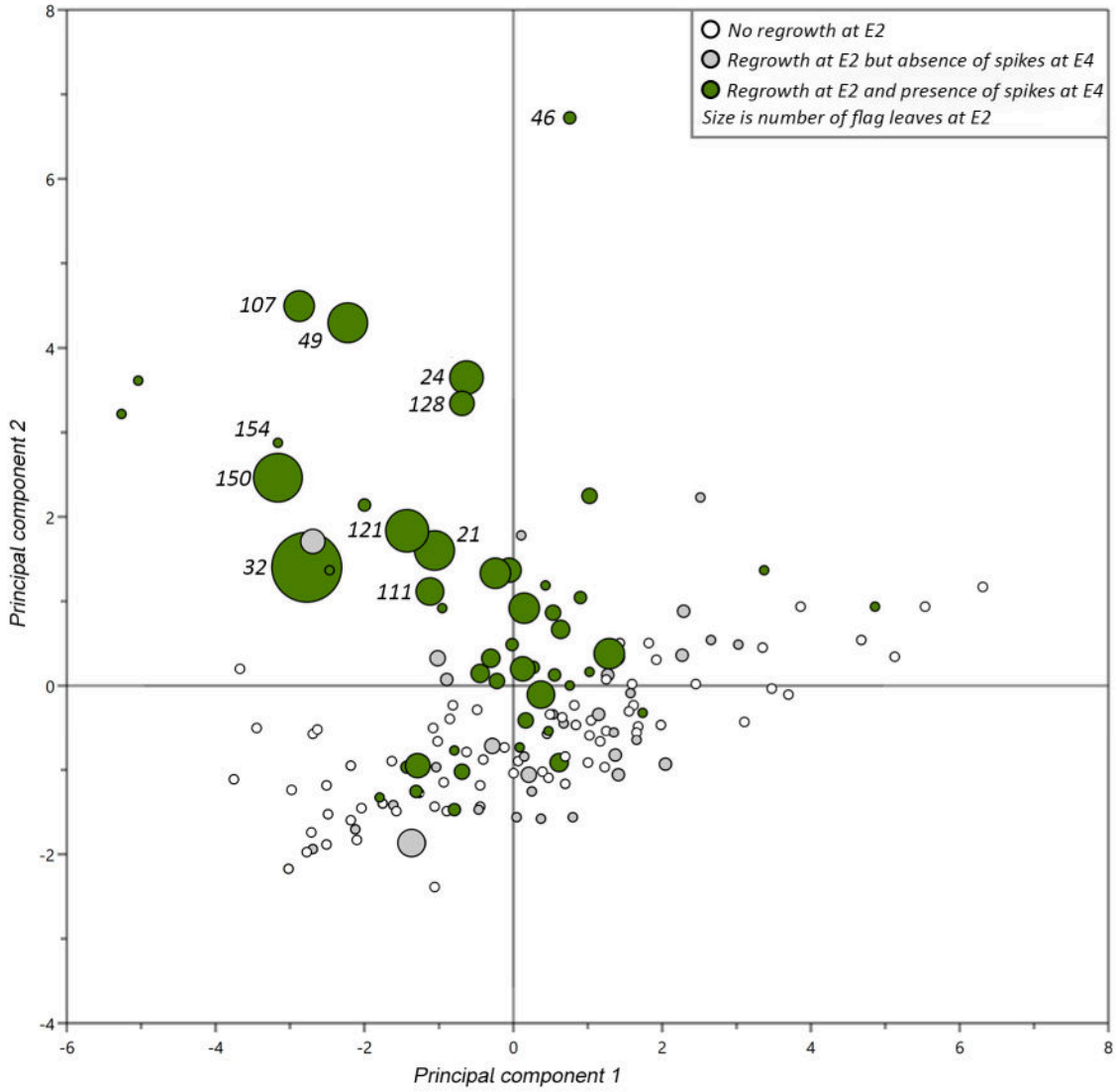

Figure 8. Principal Component Analysis, as in Figure 7 showing the distribution of ILs without regrowth at E2 (white circles), with regrowth at E2 and presence of spikes at the final evaluation (E4, green circles), and regrowth at E2 but absence of spikes at E4 (grey circles). The size of the green and grey circles is proportional to the number of flag leaves at time point E2.

3.6. Barley Parental Genetic Background Affected the Overall Phenotype of ILs

There was a clear effect of different parental genetic backgrounds on the distribution of the ILs. The ILs with an Emir parental genetic background were generally more positively associated with the growth and reproductive traits at harvest than ILs with Morex or Golden Promise as parents (Figures 6 and 7). In contrast, no clear effect of the *H. bulbosum* parental genetic background on IL distribution was observed (Figure S2).

3.7. Phenotypically Divergent ILs Were Identified Based on Vegetative and Reproductive Regrowth

The PCA revealed phenotypically divergent plants located near regrowth-related trait variables such as TNR1-c, TNR2-c, ΔTNR1-c, ΔTNR2-c, and TNR-f (Figure 7), suggesting an association with these traits and highlighting individuals with traits relevant to perennial

growth habit. Most of these ILs, exemplified by IL24, IL49, IL107, IL108, and IL128, had the Emir parental background.

3.8. Response to Planting in the Fall Under Gradually Shorter Days

Non-vernalized ILs planted in mid-August in 2015 were evaluated for the presence or absence of tillers with spikes (PSP-f) to determine their ability to flower under conditions with gradually shorter days in Field Trial 2 in fall 2015. Spike formation and flowering were observed for the parent cultivar Morex and for several ILs, particularly those having Morex as barley parent, indicating that these ILs can initiate reproductive development in the fall (Figure 9). However, approximately 40% of the ILs with the Morex background did not form spikes under these conditions. The parent cultivars Emir and Golden Promise did not form spikes in the fall, which was also the case for most ILs having them as parents. Interestingly, a phenotype opposite to the Emir parent was observed in 3% of the ILs with the Emir genetic background, indicating that these plants may be short-day responsive. These ILs had introgressions of *H. bulbosum* segments at chromosome arms 1HL, 2HS, or at both 1HL and 6HL. Regarding winter survival, none of the ILs or parental cultivars showed regrowth in the spring of the second year, as also observed in Field Trial 1.

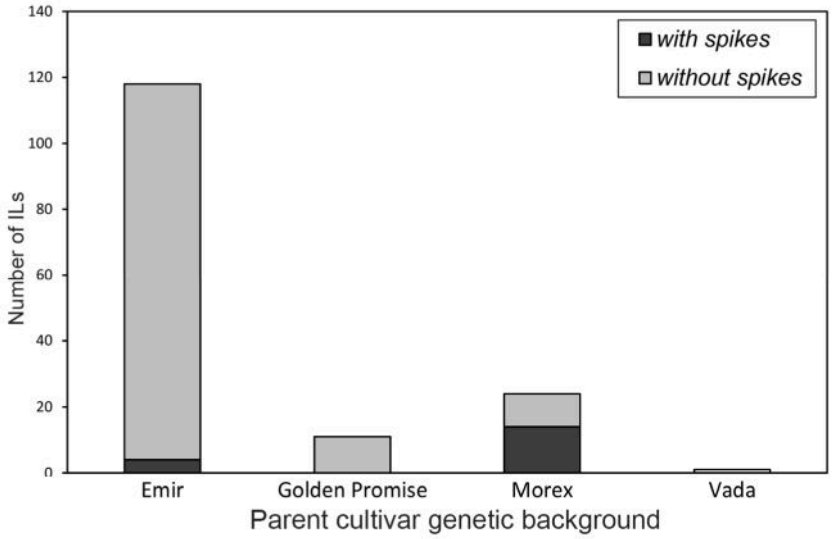

Figure 9. Number of ILs with and without spikes after growth in the fall conditions with gradually shorter days in Field Trial 2, grouped by barley parental cultivar genetic background. Plants were pre-cultivated in a greenhouse without vernalization, planted in mid-August, and evaluated at the beginning of October 2015.

3.9. Effects of Different Types of Introgressions on Regrowth

The diversity of introgressed *H. bulbosum* chromosome segments among the ILs, together with the known positions of the segments across all 14 chromosome arms [29,37], Table S1), provided a valuable opportunity to investigate the relationship between introgression type and regrowth ability. ILs with introgressions at chromosome arms 1HL and 5HL showed a comparatively high average ΔTNR-f (Figure 10). Welch's ANOVA indicated a significant overall effect of IL chromosomal group for ΔTNR-f ($p < 0.05$). Although Games–Howell post hoc pairwise comparisons did not detect significant differences, comparing group means to the overall mean by ANOM, showed that the group of ILs having introgressions on chromosome 1HL was significantly higher for ΔTNR-f. In the climate chamber, no significant overall effect was found (ΔTNR1-c, Figure 10).

Regarding specific ILs, phenotypically divergent ILs associated with regrowth traits were found across several introgression types. However, among the divergent ILs with

Emir as barley parent, none carried *H. bulbosum* introgressions at chromosomes 1H, 3H, or 7H (Figure 7). Instead, several ILs carried introgressions at 2HL (5 of 33) and 4HL (4 of 12). Single ILs had introgressions at 4HS, 5HS, 5HL, 6HS, and 6HL, and two ILs had introgressions at 2HS. Several divergent ILs with Morex as parent carried introgressions at chromosome arm 1HL (4 of 6). Single ILs had introgressions at 2HL and 4HL.

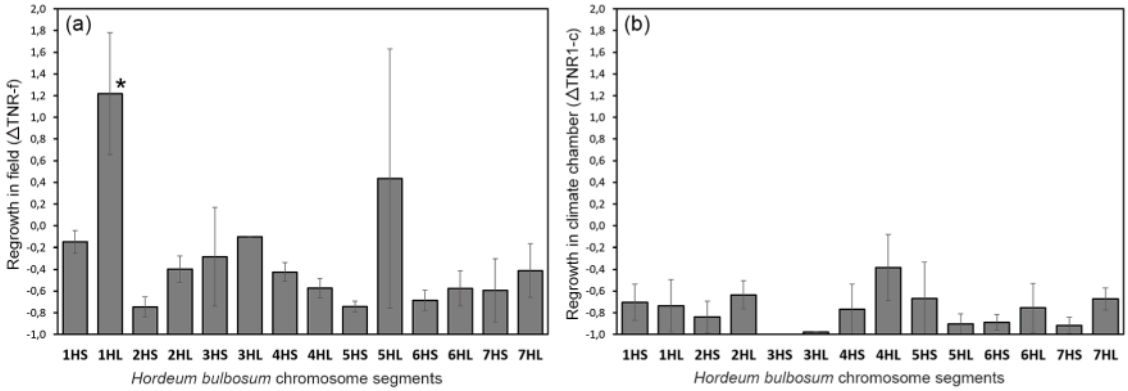

Figure 10. Regrowth after harvest in (**a**) field (ΔTNR-f) and in (**b**) climate chamber at time point E1 (ΔTNR1-f) for ILs carrying *H. bulbosum* introgressions on different chromosome arms of the barley genome. About 20% of the ILs carried more than one introgression (see Table S1), and their phenotype data were not used in the calculations. A score of −1 indicates no regrowth, while a score of 0 indicates regrowth with a tiller number equal to the number before harvest. Values are means ± SE. An asterisk indicate IL group mean is significantly different from the overall mean according to Analysis of Means (ANOM), $p < 0.05$.

4. Discussion

The two field experiments and one climate chamber experiment used in this study were valuable for evaluating barley, *H. bulbosum* ILs, for traits related to perenniality in a cold-temperate climate. The climate chamber results should be interpreted with some caution because they may be more affected by random variation among individual ILs owing to the lack of within-line replication. However, the controlled environment reduced environmental variation, making the observed differences more likely to reflect genetic differences rather than environmental effects. The correlation of tiller production before harvest for ILs in the field and in the climate chamber indicated that relative growth performance was broadly consistent during the initial growth across the two environments.

Post-harvest regrowth is an important component of the perennial growth habit because it demonstrates the ability of plants to maintain viable meristems and resume vegetative growth after reproduction. In addition, fundamental requirements of being perennial in cold-temperate climates are the ability to respond to photoperiod and temperature in fall and spring, and to develop tolerance to cold and frost. Survival and regrowth ability were clearly different in the field and in the climate chamber. In the field with lower temperatures and day-to-day and within-day fluctuations, the conditions were too stressful for any of the ILs to survive. The late winter of 2014 was, on average, milder than normal (Figure S1), commonly resulting in freeze–thaw cycles in this area. In contrast, during the simulated winter in the climate chamber, with its more stable and milder climate, almost all of the 54% of the ILs that showed regrowth after harvest in the first season continued to grow in the second growing season, and two-thirds of these ILs formed reproductive tillers (Figure 4). In addition, no correlation in tiller regrowth after the first harvest between the field and the climate chamber was observed, indicating that differences in environmental conditions between the two trials influence the phenotypic performance of the ILs at this stage. This suggests genotype-by-environment interactions, but warrants further investigation.

Evaluating the relationships among vegetative growth, regrowth, and reproductive development can enhance our understanding of perenniality and aid in the identification of ILs as genetic resources for developing perennial barley. In the field, no correlation was observed between vegetative or reproductive growth before harvest and the ability to regrow tillers before winter. Similarly, under milder conditions in a climate chamber, tiller production before the first harvest was not correlated with regrowth either before or after the cold period. In contrast, regrowth before and after the cold period was strongly correlated, and both traits showed moderate positive correlations with the production of reproductive structures, including flag leaves and spikes. These findings indicate that plant size during the first growing season did not determine subsequent regrowth capacity. Rather, genotypes exhibiting strong regrowth after the first harvest also tended to tolerate the cold period better and maintain vegetative and reproductive growth thereafter. Identifying such ILs will be valuable for the development of perennial barley.

In the context of grasses having an annual or perennial habit, the ability to show regrowth after harvest before a cold period can be a good indicator of perenniality. Annual plants complete their life cycle in one season, allocate resources to reproduction, and typically die after seed production, whereas perennials maintain structures for future regrowth [45]. The many ILs that showed regrowth after harvest, both in the field and in the climate chamber, even though they did not survive the winter in the field, may still potentially be perennial under milder field conditions. The necessity of tolerating cold and frost adds to the complexity of being perennial. The ILs that did not survive the winter conditions at the field site used in the present study may therefore have suffered from a lack of frost tolerance, but may, under milder conditions, show a perennial growth habit. These ILs are therefore valuable for further research.

The high synteny between the species [28,35–37] and a well-characterized barley genome [36] makes it possible to infer gene functions for the introgressed segments. ILs with strong regrowth were identified among several introgression types, although introgressions on chromosome arms 1HL, 2HL, and 4HL were more common. This suggests that *H. bulbosum* introgressions in these genomic regions may harbor genes contributing to perenniality-related traits. Introgressions on 3H and 7H were absent among the ILs with strong regrowth. Single or few ILs had introgressions at both arms of 2H, 4H, 5H, and 6H. In barley, the photoperiod genes *PPD-H1* (2HS), *PPD-H2* (1HL), *PPD-H3* (1HS) and *PPD-H4* (4HL), and the vernalization genes *VRN-H1* (5HL) and *VRN-H2* (4HL) are located on these chromosomes [39]. Further research should investigate the gene content of the actual ILs to show the contribution from the *H. bulbosum* parent.

Although some high-regrowth ILs carried introgressions on chromosome arms 2HL and 5HL, which are associated with cold tolerance in barley [46], they would not necessarily be expected to exhibit strong frost tolerance. *H. bulbosum* is naturally distributed in regions ranging from the Mediterranean basin to temperate Central Asia, and some of the clones used to generate the ILs are reported to originate from southern Europe. Adaptation to these relatively mild climates may explain their limited adaptation to winters with frost. Thus, while perenniality appears to be a successful strategy in the native habitats of *H. bulbosum*, the species may contribute alleles promoting regrowth rather than the level of frost tolerance required for survival under colder conditions.

In addition to the introgression type from the *H. bulbosum* parents, the barley parent cultivar also affected the regrowth traits. As cultivars, Emir, Golden Promise, and Morex differed in regrowth, with Morex lacking regrowth. ILs with an Emir parental genetic background were more positively associated with regrowth than ILs with Morex or Golden Promise as parents. Interactions between introgression type and cultivar genetic backgrounds determine regrowth. This was also clear from the field trial in which non-

vernalized plants were established in the fall under conditions with gradually shorter days. The cultivar Morex produced spikes and flowered, whereas Emir and Golden Promise remained vegetative. Morex is generally described as a spring barley with reduced photoperiod sensitivity [47], which explains why it responded to fall conditions with gradually shorter days. Control of growth and flowering is complex, and in barley is controlled by photoperiod and vernalization, regulated by *PPD* genes and *VRN* genes [47]. However, 40% of the ILs with Morex as barley parent did not flower under these short-day conditions. Morex has reduced photoperiod sensitivity due to carrying the recessive *ppd-H1* allele. Photoperiod-responsive flowering pathways appear to be conserved between *H. bulbosum* and barley. So, the *H. bulbosum* introgressions would have carried dominant alleles for photoperiod sensitivity or other genes controlling flowering. In fact, some of the non-flowering ILs with a Morex genetic background carried introgressions on chromosome arms 1HL and 4HL, where the *PPD-H2* and *VRN-H2* genes are located, respectively. *PPD-H2* has major effects on flowering time under short-day conditions, and *VRN-H2* acts as a floral repressor. In contrast to the Emir parent, 3% of the ILs with the Emir genetic background, flowered under gradually shorter day conditions. These ILs had introgressions of *H. bulbosum* segments at the chromosome arms 1HL and 2HS where *PPD-H2* and *PPD-H1* are located, respectively.

The moderate heritability (H^2 = 0.38–0.45) observed for regrowth traits in the field (TNR-f and ΔTRN-f) suggests a good response to selection for these traits, thereby facilitating the breeding of perennial barley. Our findings also suggest that several types of environmental adaptations are required for a plant to achieve complete perenniality in a cold climate. Based on the genetic and phenotypic diversity observed, some ILs, although not perennial in the tested field conditions, may carry alleles or additional genes within the introgressed *H. bulbosum* segments that are important for perenniality.

Perenniality in grasses is a complex quantitative trait controlled by multiple genetic loci and strongly influenced by environmental conditions. A defining characteristic of perennial grasses is their ability to maintain viable meristems after flowering and regenerate new tillers in subsequent growing seasons. Genes associated with perennial growth regulate axillary bud formation, tiller development, rhizome and crown development, and meristem identity. Rather than depending on a single gene, perenniality results from the coordinated regulation of meristem maintenance, post-flowering regrowth, dormancy, flowering time, and cold acclimation. Numerous quantitative trait loci (QTLs) associated with perenniality have been identified in candidate species, including sorghum, rice, and maize [48–50]. The successful development of perennial rice was facilitated by the close genetic compatibility between annual rice and its perennial relatives, enabling the production of stable hybrids. More recently, studies in rice identified the *EBT1* locus, which comprises tandem MIR156 genes, as a key regulator of perennial growth by modulating vegetative phase transitions and floral reversion, highlighting the importance of developmental regulators in controlling perenniality [51]. In contrast, initial attempts to hybridize annual wheat with intermediate wheatgrass (*Thinopyrum intermedium*) were hindered by limited genetic stability in subsequent generations [52]. As perennial growth is a complex trait requiring long-term selection, a genomic selection-based domestication strategy was chosen and it is making rapid progress [14].

The development of perennial barley is likely to require multiple complementary strategies. The ILs identified in the present study that exhibited regrowth and reproductive growth under mild conditions may represent valuable breeding resources when crossed with cold-tolerant germplasm. Sequencing the introgressed segments will be essential to identify their gene content, characterize favorable alleles, and elucidate the genetic basis of the observed phenotypic variation. Marker-assisted selection will facilitate the

retention of desirable introgressions in subsequent generations, while multi-environment field evaluations will be required to assess the stability of perennial traits. Additional genetic variation may also be introduced through crosses with domesticated perennial accessions of *H. bulbosum* [22,53].

5. Conclusions

In this exploratory study, barley–*H. bulbosum* ILs displayed substantial variation in traits associated with perenniality. Several ILs showed strong post-harvest regrowth in both the field trials and the climate chamber trial. Although none of the ILs survived the harsh winter conditions in the field, some maintained vigorous vegetative and reproductive regrowth over two consecutive growing seasons under the milder conditions in the climate chamber. The identification of introgressions on chromosome arms 1HL, 2HL, and 4HL associated with enhanced regrowth, together with the influence of the barley genetic background, highlights the complex genetic architecture underlying perennial growth. In a cold-temperate climate, not only is regrowth capacity required, but also appropriate responses to photoperiod and vernalization, as well as tolerance to cold and frost stress. Overall, the identified ILs represent valuable pre-breeding material for the development of perennial barley. Future work should focus on characterizing the introgressed genomic regions, combining regrowth-associated introgressions with cold-tolerant germplasm through targeted crosses, and evaluating the resulting material across multiple environments to assess the stability of perenniality-related traits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16151481/s1, Figure S1: Monthly mean temperatures and precipitations at the field site in Uppsala, Sweden; Figure S2: Principal Component Analysis illustrating the distribution of ILs with different *H. bulbosum* genetic background; Table S1: Barley—*Hordeum bulbosum* introgression lines included in evaluation of regrowth in field and climate chamber, and their introgression types based on [29,37,54,55].

Author Contributions: Conceptualization, A.W., M.S., E.L.K. and P.-O.L.; methodology, A.W., M.S., E.L.K. and P.-O.L.; formal analysis, A.W. and P.-O.L.; investigation, M.S. and E.L.K.; data curation, A.W. and P.-O.L.; writing—original draft preparation, A.W. and P.-O.L.; writing—review and editing, A.W. and P.-O.L.; visualization, A.W. and P.-O.L.; project administration, A.W.; funding acquisition, A.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in this article; further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declare no conflicts of interest.

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